THE PRODUCTION OF γ-LINOLENIC ACID BY *CHOANEPHORA CURCUBITARUM*

by

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Submitted in partial fulfilment of the requirements for the degree of M.Sc. in the Faculty of Science University of Cape Town Rondebosch

SEPTEMBER 1985

ACKNOWLEDGEMENTS

I wish to thank my supervisor, Professor David R Woods, for his encouragement, enthusiasm and guidance throughout this project. Sincere thanks, too, to all at National Chemical Products for their support and technical assistance.

I wish to acknowledge the research bursary awarded to me by the CSIR, South Africa.

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ABSTRACT

Gamma-linolenic acid (GLA) is an essential fatty acid which has been shown to be beneficial for a large number of diseases. At present the fatty acid is marketed as a health product and the prime source of GLA is the evening primrose flower. There are several problems associated with producing the oil and as there is a growing demand for GLA for pharmaceutical and clinical applications, alternative sources are being sought. Choanephora curcubitarum is known to produce GLA and the production of GLA by fermentation of this organism has been successfully achieved by National Chemical Products. The ultimate aim of this project is to genetically manipulate this organism in order to increase the yields of GLA. In this thesis the basic groundwork needed to develop a genetic system in C. curcubitarum has been covered.

The first part of the study deals with the extraction and analysis of the mycelial lipid. The conventional methods of extraction and analysis are tedious and time consuming and not at all suitable for screening for mutants in lipid production. A rapid method for the extraction and analysis of bacterial fatty acids has been quantified and adapted for the analysis of the mycelial lipid. The variance and the validity of the method was examined and the method was compared to a

more conventional method of extraction and analysis developed by researchers at National Chemical Products. The rapid method developed was termed the "HP" method of extraction and analysis and was found to be the method of choice. A system was developed whereby C. curcubitarum mutants in GLA and lipid production could be screened using this method and was found to be successful.

The lipid extracted from C. curcubitarum was fractionated in order to determine in which fraction of the cell the GLA was found. The fatty acid was present mainly in the triacylglyceride fraction of the lipid and would, therefore, be found mainly in lipid globules which are clearly visible in the mycelium.

The second part of the study deals with the factors affecting GLA and lipid production by C. curcubitarum. The age of the culture, the carbon to nitrogen ratio (C:N ratio) in the medium, the temperature, pH, and aeration were found to markedly influence the GLA and lipid yields. Lipid accumulation followed a pattern of lipid accumulation and depletion, reaching a maximum after 14 days of incubation in liquid medium cultures and after 4 days of incubation on agar medium.

The optimum environmental conditions for GLA production by C. curcubitarum were: a C:N ratio of between 20:1 and 30:1; an incubation temperature of 30'C; a pH of between

5.5 and 6.0; and maximum aeration of the medium during incubation. Shaw's agar medium was found to be optimum for GLA production by the organism.

It was decided to investigate the possible link between carotogenesis and lipid production. If a link was found, the colour difference could be used as a simple screening technique (the mutants would be easily distinguishable from the wild-type, WT, on the assay medium). However, the two proccesses proved not to be directly linked, even though a few mutants were isolated which were selected on this basis.

The third part of the study investigates the induction of mutations and the isolation of C. curcubitarum mutants with increased GLA production. A suitable medium for assaying the fungal colonies was developed. Exposure of the spores to 0.1 mg/l NTG in distilled water for 30 - 60 mins resulted in 0.1 - 10 % survival of the spores. Staining the lipid globules in the mycelium was investigated as a possible method for screening for mutants in lipid prodution, but was not successful.

Four mutants in GLA production were isolated, but these were unfortunately not stable. The link between the increase in GLA production and increased carotene production in these mutants was co-incidental and could be due to multiple mutations induced by NTG.

CHAPTER 1

GENERAL INTRODUCTION

1.1 CLINICAL SIGNIFICANCE AND PRESENT SOURCE OF GLA

In recent years there has been an upsurge of medical interest in the essential fatty acid (EFA), gamma-linolenic acid (GLA). The clinical significance of GLA has been reviewed by Graham (1984) and the clinical information, unless otherwise quoted, has been extracted from this book.

GLA is reported to be beneficial in a number of diseases such as multiple sclerosis, benign breast disease, heart disease, vascular disorders, high blood pressure, eczema, cystic fibrosis, acne, asthma, rheumatoid arthritis and other inflammatory diseases, schizophrenia, alcoholism and cancer, and in conditions such as obesity, premenstrual syndrome, allergies and hyperactivity in children. The connection between GLA and cancer is being investigated because one of the differences between a normal cell and some types of cancerous cells is that the cancer cell does not manufacture GLA. Although certain researchers (Horrobin, 1978 & 1980a; Dippenar *et al.*, 1982a & b; Leary *et al.*, 1982) consider GLA to be important in cancer, the exact position is contraversial and unclear at present and

futher vigorous research needs to be done before the role of GLA in cancer is understood. Further research is also being done on many other related diseases. The only side effects that have thus far been noted are loosening of the stool and headaches which occur in 1 % of patients (Horrobin, private communication).

This may seem to be an unrealistically long list of diseases for which GLA may be helpful, but if one were to examine the role of GLA in the body, the significance of this EFA becomes clearer.

EFAs have many functions in the body and are an essential part of nutrition. They provide energy, help maintain the body temperature, insulate nerves, and cushion and protect the tissues. Roughly 60 % of the brain is made up of lipids, of which the essential fatty acids are an important part and are vital for the proper growth and development of the brain and central nervous system. The EFAs are incorporated into all cell membranes, mainly as complex lipids and a deficiency would lead to abnormality of all cells in all tissues and organs (Booyens, 1983). They, therefore, also have an influence on the fluidity and flexibility of the lymphocytes, which are a vital part of the immune system. The EFAs are also the metabolic precursors of the all-important prostaglandins and related compounds as shown in Fig. 1. The prostaglandins are amoungst the most prevalent autocoids in the body and are found in

almost every tissue and body fluid, where they have a vital regulatory function. They are very short lived molecules and are rapidly destroyed by the body within a matter of seconds. No other class of autocoids show more numerous and diverse effects and those who have reviewed the field have remarked on this "awesome" and "bewildering" diversity (Douglas, 1975). More than 50 prostaglandins, thromboxanes and leucotrienes have been identified and new ones are discovered annually (Booyens, 1983).

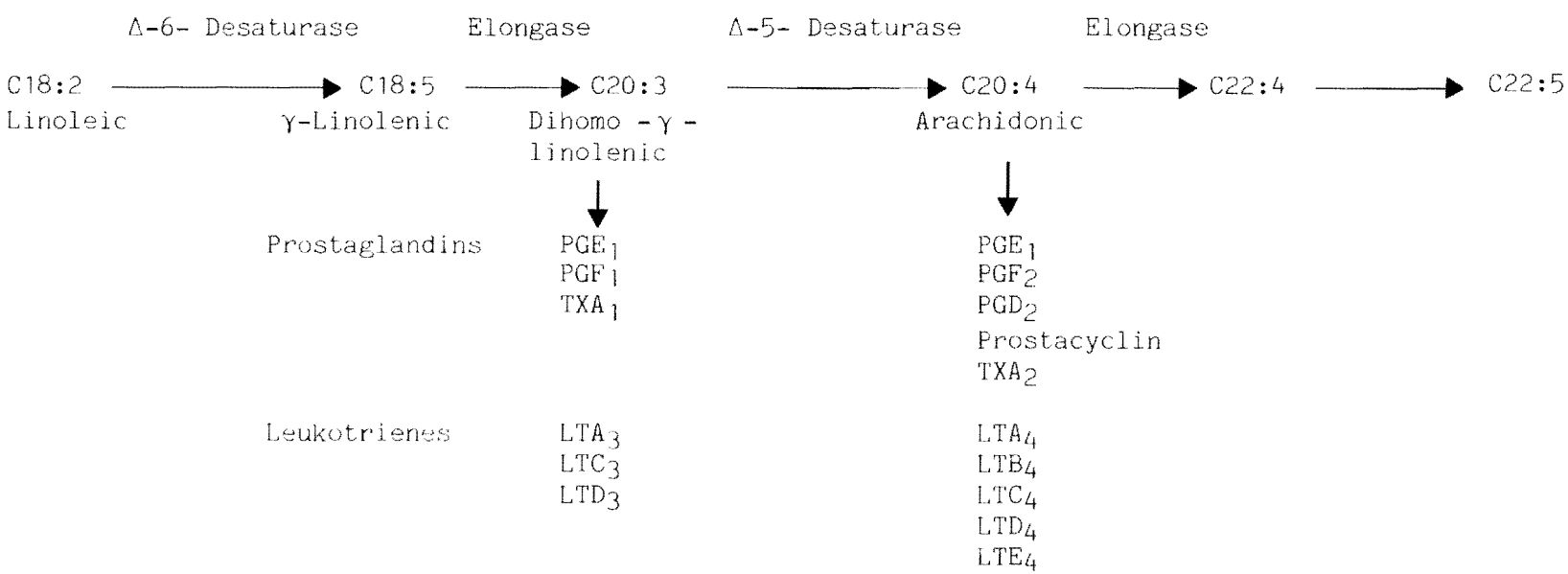
GLA is converted into the prostaglandin, PGE1 (Fig. 1). PGE1 is known to have the following desirable properties (Douglas, 1975):

- It is a potent vasodilator and has vasodilatory effects on coronary and other human vascular beds.
- It lowers blood cholesterol levels
- Increases cardiac output (PGE series in general)
- Decreases systemic blood pressure and blood flow to most organs, including the heart and kidney, is increased (PGE series in general)
- Relieves angina
- Is a potent bronchodilator
- Inhibits gastric secretion
- Stimulates lipolysis
- Heals skin ulcers (Olsson, 1978) and varicose leg ulcers (Horrobin, private communication).
- Activates defective T-lymphocytes

- Potent natural inhibitor of platelet aggregation and prevents thrombosis
- Inhibits inflammation (Horrobin, 1980; Fantone, 1980)
- Inhibits abnormal cell proliferation (Bregman, 1982)
- Prevents alcohol withdrawal symptoms and prevents liver damage in alcohol addicted animals
- PGEs infused directly into the renal arteries of dogs increase renal blood flow and provoke diuresis, natriuresis and kaliuresis

The 2 series of prostaglandins are formed from arachidonic acid and most of this EFA is obtained from meat and some dairy products (delta-5-desaturase (Fig. 1) has a limited function in adults). Arachidonic acid is stored in the body as phospholipids and is made available to the body by enzymatic cleavage. The free arachidonic acid is then converted into prostaglandins, thromboxane and hydroxyacids and leucotrienes (the latter two compounds being potent broncho-constrictors). These compounds have very mixed actions, some of which are desirable and others are highly unfavourable. For instance, PGE2 is associated with the inflammation reaction in the body, causing pain and discomfort. PGE1 has a steroid-like action in blocking the release of arachidonic acid from the tissues and therefore the levels of the inflammatory PG2s are actually lowered. PGE1 also has a direct control on the action of TXA2

Fig. 1: Derivation of prostaglandins and leuktrienes from essential fatty acids (Horrobin, private communication)



which is the most potent vasoconstrictive and pro-aggregator compound known (Horrobin, 1980b).
Linoleic acid is the main EFA in the diet and naturally occurs in the "cis" configuration. Trans-linoleic acid has no EFA activity and in fact it acts as an anti-vitamin, increasing the requirement for the true essential fatty acids (Kummerow, 1979). Linoleic acid in the diet is converted by the enzyme delta-6-desaturase to GLA. If this enzyme is blocked, then sufficient arachidonic acid will most likely be obtained from the diet and the main defects will be in the GLA, DGLA and the PG1 series of prostaglandins. The enzyme is known to be blocked by the following factors: the aging process (Horrobin, 1981), foods high in saturated fats, cholesterol, trans fatty acids (which form during the processing of vegetable oils), alcohol, viral infections, diabetes mellitus, excessive glucose or starvation (Booyens, 1983), adrenalin, glucocorticoids, radiation, cancer and Zn deficiencies. By taking GLA the metabolic step, where this enzyme is required, is bypassed and the body can then proceed further along the pathway to manufacture the beneficial PGE1.

It is interesting to note that mothers milk contains high % of linoleic acid and GLA (Gibson, 1981).

At present the most important commercial source of GLA is the oil extracted from the seeds of the evening primrose (Oenothera biennis or Oenothera lamarkiana)

which contain up to 9 % GLA. Capsules of the oil are marketed all over the world as a health product (eg. Efamol G or Naudicelle). There are, however, a number of problems assosiated with growing crops of the evening primrose. The plant is a biannual and therefore has a two-year cycle, the seeds are very small and thus difficult to harvest and extract, and yields are subject to uncontrollable conditions such as the weather and to disease (Horrobin, private communication). Alternative sources are therefore being sought to meet the growing demand for clinical and pharmaceutical applications.

1.2 LIPID PRODUCTION BY MICRO-ORGANISMS

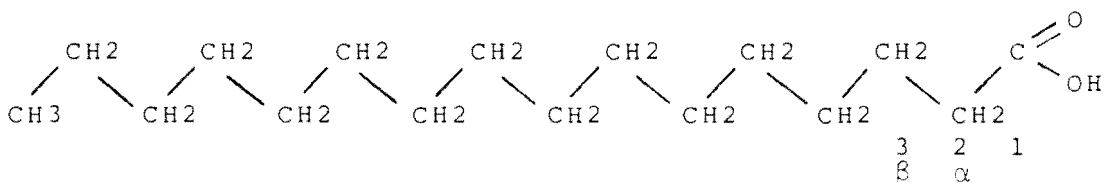
Certain micro-organisms possess a high capacity to form and accumulate triacylglycerol and are referred to as "oleaginous" micro-organisms. These organisms produce 25 % or more of their biomass as lipid. "Lipid" generally refers to the total material which can be extracted from a source with a solvent such as chloroform and encompasses all substances having an aliphatic hydrocarbon chain and also substances which are soluble in these materials (Ratledge, 1982). This includes sterols, terpenes, carotenoids, tocopherols and other fat soluble vitamins which do not have a fatty acyl group associated with them. Whether the terms "lipid", "fats", or "oils" are used it refers to a mixture of materials.

The triacylglycerols and fatty acids in the lipid extract are of principal commercial interest. Fatty acids, the most abundant class of lipid in nature, are monocarboxylic acids containing a straight, branched or cyclic hydrocarbon chain of variable length. They are not usually found as free molecules but are normally bound as esters to glycerol (triacyglycerides), long chain amino alcohols, sugars or sterols as they would be too toxic to be contained within the living cell. The triacylglycerols usually comprise approximately 95 % of all plant and animal oils and their properties are governed by the constituent fatty acids and the

position these fatty acids take up on the glycerol molecule (Ratledge, 1982).

Fatty acid nomenclature has been reviewed by Weete (1974a) and the nomenclature used in this thesis has been summarized below.

Both trivial and systematic names for the fatty acids are found throughout the scientific literature. The systematic naming of fatty acids is consistent with the naming of organic compounds. The longest hydrocarbon chain is indicated by the appropriate Latin term to denote the number of carbon atoms and the suffix "-oic" is used to represent the carboxyl function. For example, a fatty acid which contains 16 carbon atoms in a straight chain is called palmitic acid and is systematically named hexadecanoic acid. This acid may be abbreviated by the following symbols: C16 or C16:0. The position of the substituents along the hydrocarbon chain is indicated by the carbon number of its place of attachment beginning with the carboxyl carbon as number 1 and the subsequent carbon as 2 or alpha.



Two methods are used to designate the position of the double bond along the fatty acid molecule. Unsaturation may be designated by the number of the first carbon atom

from the carboxyl end of the molecule which is involved in the double bond structure. For example, a C18 diunsaturated fatty acid with double bonds in the 9 and 12 possitions may be designated by Δ9,12 oxtadecanoic acid and abbreviated C18:2 (Δ9,12). This fatty acid is commonly known as linoleic acid or linoleate. GLA may be designated by Δ6,9,12 octadecatrienoic acid and abbreviated C18:3 (Δ6,9,12). Unsaturated fatty acids may occur in the cis or trans configuration. The distribution of the double bonds along the hydrocarbon chain may be of two types: "methylene interrupted" and "nonmethylene interrupted".

The methylene interupted arrangement of the double bonds, which is the most common polyene of structure of naturally occuring fatty acids, is a 1,4-diene nonconjugated double bond system:

R-CH=CH-CH2-CH=CH-R

The nonmethylene interrupted double bond arrangement is illustrated by the conjugated double bond system or when two double bonds are separated by the two or more methylene groups in the hydrocarbon chain. Unsaturated fatty acids can also be grouped into families according to the double bond positions relative to the terminal methyl group and the mode of biosynthesis. These families are designated Δ9, ω-9, ω-6 and ω-3. GLA belongs to the ω-6 familiy and alpha-linoleate to the ω-3 family.

Diversity in type of fatty acids that have been detected in various living systems suggests that different lipid properties are required by each organism for survival. The differences are mainly in the chain length, saturation and substitution. In bacterial systems, fatty acid chain lengths are usually from C10 to C20, with predominance of those with 15 to 19 carbons. In fungi, there is a abundance of C16 and C18 fatty acids, with oleic and linoleic as the major fatty acids. In animals and plants, besides the fatty acids found in fungi, various fatty acids with chain lengths of C20 and higher may be found. In higher eucaryotes the fatty acid composition is fairly constant and any variations are slight. Micro-organisms on the other hand have wide variation in fatty acid types and show characteristic differences.

1.2.1 LIPID PRODUCTION BY FUNGI

Fungi appear to have the most potential to produce polyunsaturated fatty acids (Wassef, 1978; Ratledge,1984), although oleaginous fungi have not been extensively studied (Ratledge, 1982). The lipid content of vegetative hyphae varies between 1 and 50 % of the dry weight, depending on the species, developmental stage of growth and the cultural conditions. The developmental stage of growth and cultural conditions have been dealt with in chapter 3.

Lipid globules in the mycelium are thought to consist primarily of triacyglycerides and function as an energy reserve. Lipids, however, not only serve as a reserve energy source but have more specific roles in cellular growth and reproduction. They are, for example, essential components of the membrane structure and function (transport), and may act as stimulators of growth and reproduction or as protective coatings (Weete, 1974a). The function of polyunsaturated fatty acids has been reviewed by Shaw (1966c). Based on the production of GLA or alpha-linoleate, Shaw suggested that fungi can be sharply divided along taxanomic lines. However, analysis of the fatty acid composition of Phycomyces show that this hypothesis is only applicable to the order Mucorales (Wassef, 1978). The role of GLA has been discussed in chapter 3.

The total lipid in fungal spores varies from species to species, but ranges between 1 and 35 % of the spore dry weight. The ability of spores to germinate without an exogenous carbon source appears to be related to the abundance of reserve lipid (Weete, 1974a). Sumner and Morgan (1969) found that there was qualitative and quantitative similarities between the spores and parent mycelium in Mucor and Rhizopus. The % of unsaturation was, however, lower in the spores.

The Phycomycetes (lower fungi) are known to produce GLA while other higher fungi produce the alpha-linolenic

acid isomer (Wassef, 1978). The total lipid content and fatty acid compostion of fungi has been reveiwed by Shaw (1966), Weete (1974b), Wassef (1978) and Ratledge (1982). The strains reported to produce more than 10 % GLA in the total fatty acids were investigated and their GLA yields determined by National Chemical Products. The highest yield of GLA was obtained from Choanephora curcubitarum 12997, which produced 204.98 mg/l GLA in a Chemap laboratory fermentor using Shaw's medium (see appendix) at 28°C.

The environmental factors affecting fat production by fungi have been revieved in chapter 3 and the optimum environmental conditions for GLA production by C. curcubitarum were determined. The environmental factors have a marked influence on fatty acid production by the organism.

Not much research on C. curcubitarum has been published. It belongs to the class Zygomycetes, order Mucorales. In nature the fungus is a saprobe and may be found on squash blossoms and fruit where it can cause considerable damage (Alexopoulos, 1962).

C. curcubitarum produces both multispored sporangia and monospore sporangiola (conidia). Barnett and Lilly (1950 & 1955) studied the influence of nutrition and environmental factors (temperature, light, carbon dioxide and humidity) on the sporulation of C.

curcubitarum. The production of conidia was found to be influenced by the the amounts of sugar and thiamine in the medium. High temperature (30-31°C) and high relative humidity favoured sporangial production, while lower temperatures (25°C) and low relative humidity favoured conidial production. Light was found to have little or no effect on the mycelial growth but the formation of conidia was governed by light conditions. The cultures had to be exposed to conditions of alternating bright light and darkness or to continuous light of low intensity for conidial formation. The accumulation of carbon dioxide in closed vessels reduced or prevented sporulation.

C. curcubitarum is a heterothallic fungus (Wu and Chien, 1980) but few studies have been undertaken to determine the optimum conditions for sexual reproduction. Barnett and Lilly (1956) found that zygospores formed under a much wider range of conditions than asexual spores. Light, temperature, pH and the CO2 concentration was found to have little effect on zygospore formation (within limits). Glucose starvation favoured sexual reproduction. Wu and Chien (1980) found that different media affected zygospore formation in Choanephora.

Lipid globules and carotene can be easily seen in the mycelium of C. curcubitarum when examined using a laboratory microscope. The work published on lipid production in C. curcubitarm has been reviewed in

chapter 3. Seven major fatty acids were identified by researchers at National Chemical Products. These are: myristic acid, palmitic acid, palmitoleic acid, stearic acid, oleic acid, linoleic acid and gamma-linolenic acid or GLA.

1.2.2 FATTY ACID BIOSYNTHESIS

Fatty acid metabolism in fungi has been reviewed by Weete (1974c) and Chopra and Khuller (1984) and the following information, unless otherwise quoted, has been extracted from these reviews.

Lipid synthesis has been studied extensively in bacteria, plants and animals, but information on the lipid synthesis in fungi is mostly compositional. It is generally assumed that what is true for higher eukaryotes holds true for lower eukaryotes. This may be misleading, however, as metabolic differences exsist between various tissues in a plant and even between species of the same genus. Only a few areas of lipid metabolism in fungi have been investigated and in detail, while other areas have been largely ignored.

There are two sets of enzymes involved in fatty acid synthesis. The first set is acetyl-CO-A carboxylase and the second is the fatty acid synthetase complex. Acetyl-CO-A carboxylase provides malonyl-Co-A which is required for the initiation of fatty acid synthesis by

condensation with acetate units. The condensation, elongation and ultimate release of the fatty acid are catalyzed by the synthetase complex.

The overall reaction of fatty acid synthesis can be represented by the following expresssion:

$$\text{Acetyl-Co-A} + n\text{Malonyl-Co-A} + 2nH^{+} \rightarrow$$
$$CH_3(CH_2\text{-}CH_2)\text{-CO-CoA} + nCO_2 + n\text{CoA} + 2n\text{NADP} + nH_2O$$

This sequence of reactions is responsible for producing the fatty acids of variable chain length (C12-C18) present in most biological systems. The principal products of these reactions are, however, palmitic acid (C16) and stearic acid (C18).

The first reaction, initiating the synthesis of fatty acids, is catalyzed by acetyl-CoA carboxylase and results in the formation of malonyl-Co-A from acetyl-Co-A. This enzyme has been studied in many organisms including yeasts and plants. In eukaryotes, the enzyme exists as an aggregated complex and dissociation of the complex into protomeric structures renders it inactive. The individual reactions catalysed by acetyl-CoA carboxylase have been investigated in Escherichia coli as the enzyme of this bacteria remains active after dissociation.

The first subunit of the acetly-CoA carboxylase in E. coli, biotin carboxylase, carboxylates the biotin moeity

in the biotin carrier protein (BCCP). BCCP acts as a carrier of biotin from one enzyme to the other during the reaction catalysis. Transcarboxylase, the third subunit, reversibly transferes the carboxyl group from the carboxylated biotin of BCCP to acetyl- CoA, forming malonyl-CoA. In yeast and animal enzymes, a fourth subunit, the citrate-binding unit, binds citrate which is an activator of acetyl-CoA carboxylase.

The reaction catalyzed by acetyl-Co-A carboxylase proceeds in two steps:

$$ATP + HCO3^- + BCCPP \xrightarrow{Mn^{2+}} CO2\text{-}BCCP + ADP + Pi \quad (1)$$

$$CO2\text{-}BCCP + Acetyl\text{-}CoA \Longleftrightarrow Malonyl\text{-}CoA + BCCp \quad (2)$$

The first reaction is catalysed by biotin carboxylase in the presence of Mn^{2+} at the expence of an ATP molecule. The second reaction is catalysed by transcarboxylase and results in the formation of malonyl-CoA. Acetyl-CoA carboxylase of Candida lipolytica is stabilized by glycerol and is stimulated by polyethylene glycol instead of citrate in yeast.

Regulation and content of acetyl-CoA carboxylase varies with dietry, hormonal, developmental and genetic conditions. Acetyl-CoA carboxylases, in general, are inhibited by long chain acyl-CoA (particularly palmityl-CoA). In the presence of palmityl-CoA, the supply of malonyl-CoA becomes rate limiting, thereby inhibiting the synthesis of fatty acid in the organism. Membrane

phospholipids may also regulate the activity of this enzyme.

The process of fatty acid synthesis is completed by the second set of enzymes, fatty acid synthetase (FAS). These enzymes occur in an aggregated complex in animals and fungi, whereas plants and bacteria usually have a non-aggregated synthetase (although there are exceptions). The FAS in E. coli are not tightly grouped and have been investigated in some detail due to the easier determination of reaction intermediates. The different reactions of the FAS in are represented in Fig. 2. The reactions involve the transfer of the primary reactants from CoA to acyl carrier protein (ACP), condensation, two reductions and dehydration. When the prefered chain length is produced, a terminal transfer reaction occurs which gives rise to the final fatty acid product.

Fatty acids are synthesized in a similar manner in all organisms and ACP serves as a central carrier of the acyl chain. Minor differences have, however, been noticed from one system to another.

The terminal reaction is catalysed by Palmityl-ACP thioesterase and the enzyme bound products are removed from ACP of the synthetase by either 1) transfer to free ACP, 2) transfer to CoA, or 3) hydrolysis to the free fatty acid.

The synthetases produce chain lengths no longer than 18 carbons, yet fatty acids of longer chain lengths are found in fungi as well as plants and animals. Fatty acid elongation in animal, plant and bacterial systems which utilize malonlyl-CoA (located in the microsomes) and acetyl-CoA (mitocondrial) has been described. In animal and bacterial systems, chain elongation has been suggested to be the reversal of the β -oxidation process as shown in Fig.3. There is, however, a difference between the two processes: in the terminal enzyme of the elongation reactions and the initial enzyme of the β-oxidation system. For β-oxidation, the process moves towards the thiolase-catalysed reaction, whereas for elongation it is towards the enoyl-CoA reductase reaction.

Lipids produced by most biological systems generally contain a high degree of unsaturation which is due to monoenoic and polyenoic fatty acids present in their structures. Monounsaturated fatty acids are produced by all living organisms and with the exception of bacteria, polyunsaturated fatty acids are also widely distributed in nature. There are two known pathways of fatty acid unsaturation, one being aerobic and the other anaerobic. The aerobic pathway operates mainly in eukaryotic living systems, while the anaerobic is restricted to prokaryotes. The enzymes involved in the desaturation process are known as the desaturases.

Fig.2: The pathway of saturated fatty acid biosynthesis, catalised by the fatty acid synthetase (FAS) complex of enzymes (Weete, 1974c).

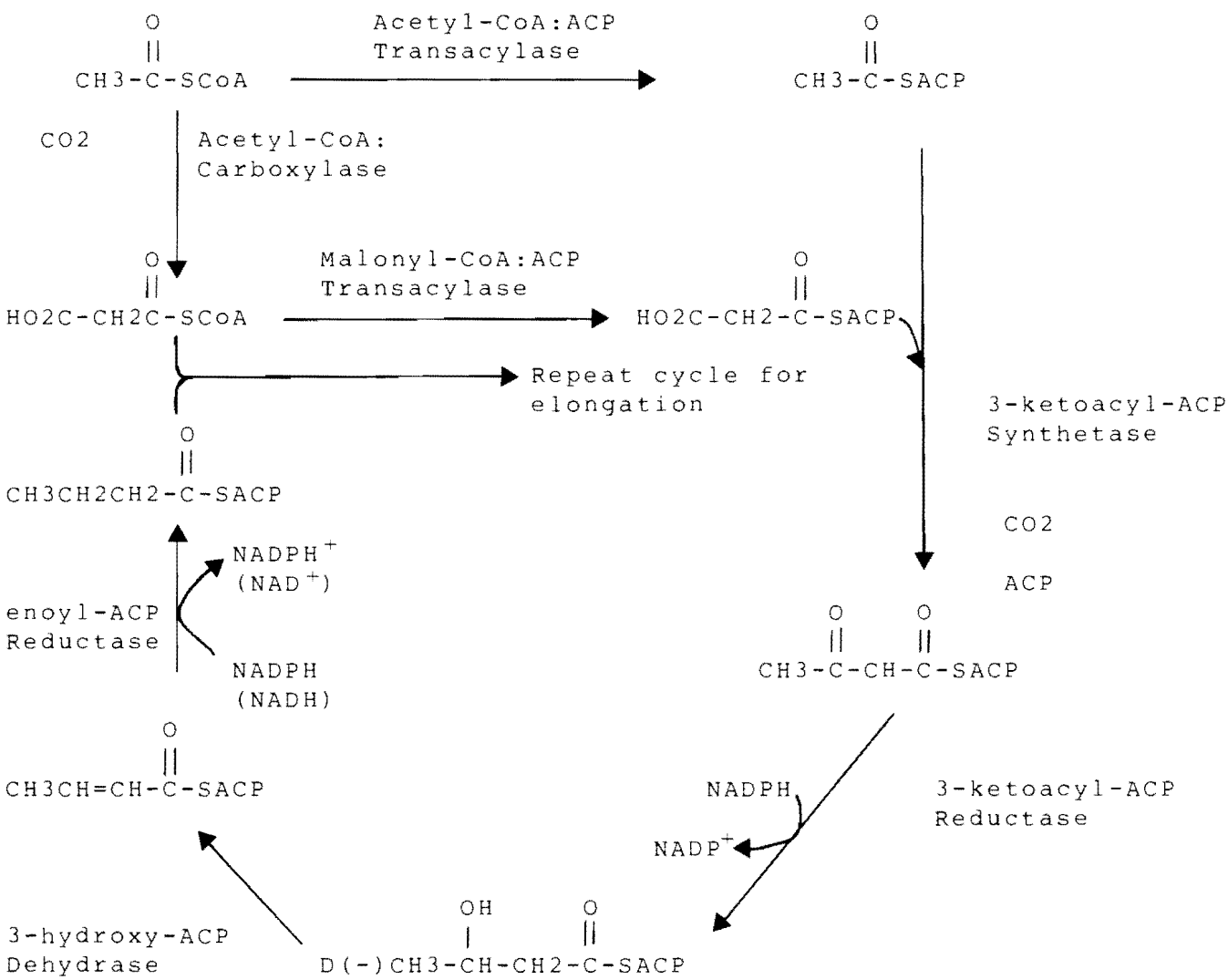


Fig. 3: Elongation and ß-oxidation of fatty acids (Chopra and Khuller, 1984).

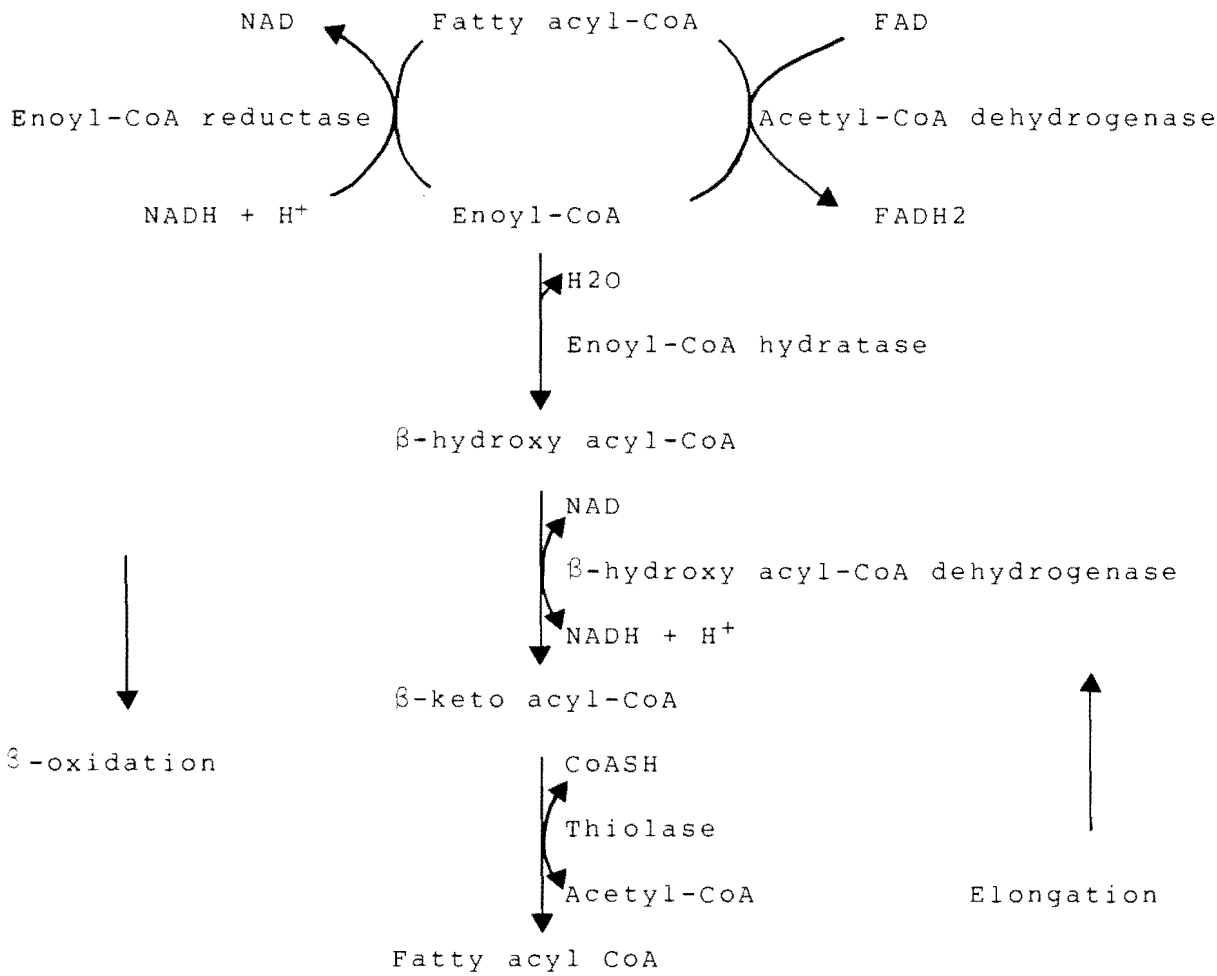
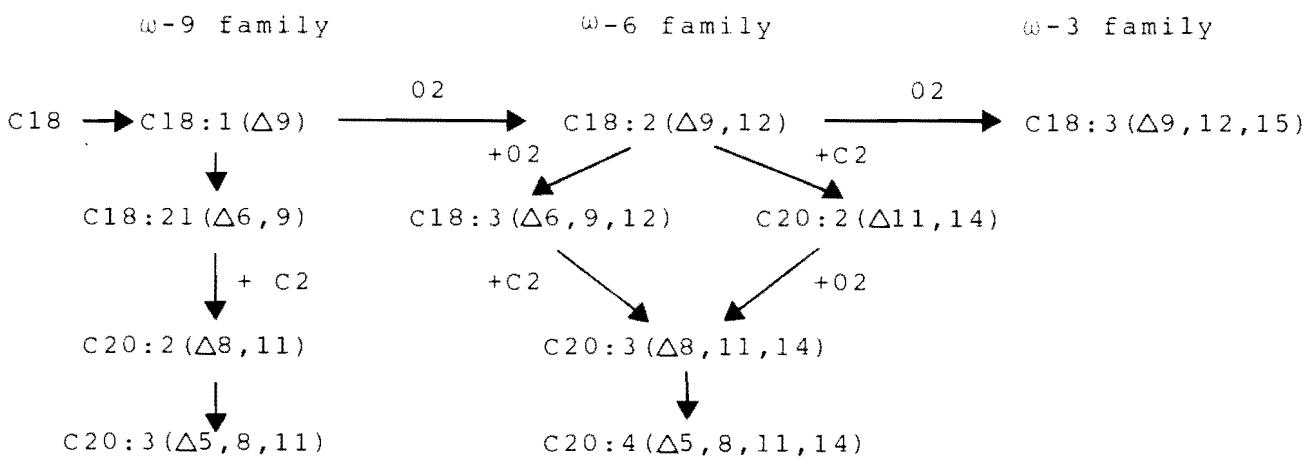
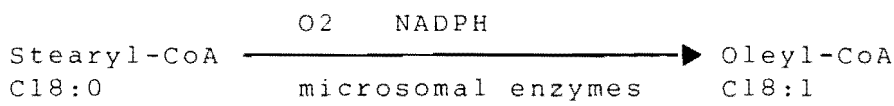


Fig.4: Principal pathways of polyunsaturated fatty acid biosynthesis in plants and animals (Weete 1974c).



The desaturase system found in animals, fungi and bacteria appears to be an aggregate of enzymes associated with the microsomal fraction of the cell. The desaturase system from hen liver contains three components: (1) a desaturase, (2) NADH cytochrome b5 and (3) cytochrome b5. Thiolesters (CoA) of C16 and C18 fatty acids are desaturated by this system to their respective Δ-9-monoenes. Reduced $NADP^+$ and molecular oxygen are required in the desaturation process:



The desaturase system in the mycelial fungus Neurospora crassa was reported to be similar to that of yeast and animals. Stearyl-CoA is rapidly converted to its delta-9 unsaturated isomer by the enzymes of the microsomal fraction of the fungus. Both NADPH and NADP are effective electron donors for this system, but there is a pronounced preference for the latter. It was shown that the acyl-CoA substrates are tightly bound to the microsomes and are available for several competing reactions: (1) nucleotide-O2-dependent desaturation, (2) gylcerophosphatide formation, (3) triacylglyceride formation and (4) deacylation. A similar type of desaturase has been found in Penicillium chrysogenium. The exact mechanisms involved in the dehydrogenation processes are not well established. The desaturase is highly specific for introducing a double bond in the

9,10 position of fatty acid thiolesters with 18 of less carbon atoms. The absolute specificity of desaturases probably arises from the enzyme binding the activated carboxyl end of the substrate, which allows for the 9 and 10 methylene groups to be properly positioned at the active centre. A second delta-9-desaturase, specific for short chain fatty acids, may be present in some species. A yeast system has, however, been reported to desaturate at delta-9 in small chain fatty acids such as C10 as well as C16 and C18 fatty acids. It was suggested that the appearance of certain monoenes in a species is not governed by the specificity of the desaturase, but by the specificity of the FAS which provides the acyl-thioester substrates. The most common monoenes are palmitoleic acid (C16:1) and oleic acid (C18:1).

There are three desaturases; the delta-9-desaturase is responsible for introducing the first double bond into the fatty acid and the delta-6-and delta-5-desaturases are involved in the polydesaturation (Gurr and James, 1971; Okayasu, 1981). Generally, the polyunsaturated fatty acids have the cis-configuration and are of the methylene interupted type. The most common polyenes are linoleic [C18:2 (Δ-9,12)], alpha-linolenic [C18:3 (Δ-9,12,15)] and gamma-linolenic [C18:3 (Δ-6,9,12)].

Polyunsaturation proceeds via sequential desaturation of oleic acid and chain elongation. The principal pathways

of polyunsaturated fatty acid biosynthesis in plants and animals has been summarised in Fig. 4. The double bonds are introduced into the molecule in one of two ways. In plants, desaturation occurs between the original central delta-9 double bond and the terminal methyl group and is referred to as ω-3type desaturation. In this pathway alpha-linoleate is produced via linoleate.

Higher animals cannot produce polyunsaturated fatty acids by terminal methyl-directed desaturation and cannot therefore synthesise linoleic acid, which explains why this fatty acid is an essential fatty acid in humans. Desaturation is carboxyl-directed.

The ω-6 family is produced by some animals and and lower organisms. Of particular importance is arachidonic acid, C20:4 (Δ-5,8,11,14), which may be produced by two pathways. The predominant pathway in animals and certain algae involves the route via chain elongation to C20:3 (Δ-8,11,14) followed by carboxyl-directed desaturation.

Generally, the alpha-linoleate pathway is characteristic of plants and the gamma-linoleate pathway is characteristic of animals. The lower forms of life may, however, contain characteristics of both pathways.

The mechanisms of polyene formation are thought to be similar to that of monoene formation. In fungi, the coenzyme A thioester of oleate is used as the principal substrate for the desaturase enzyme and molecular O2 and

NADH or NADPH are required as co-factors. Evidence is accumulating suggesting that polar lipids such as phosphatidylcholine also act as substrates for oleate desaturation in various organisms, including fungi.

Two types of desaturases were detected in Candida lipolytica. The first type was more active on oleyl-CoA and was therefore termed a fatty acid desaturase. The second was more active on phospholipid acyl chains and was termed phospholipid desaturase. From studies on this fungus it was concluded that direct desaturation of phospholipids can take place. The fatty acid desaturase was suggested to regulate the phospholipid composition since its activity would make different unsaturated fatty acids available.

The presence of two desaturase systems in Neurospora has been postulated as production of C18:2 and C18:3 fatty acids was observed to take place by two different routes, each indpendent of each other.

The precise mechanism of desaturase activity is uncertain not only in fungi, but in plants and animals as well. Regulation of desaturase activity is important for microbial membrane functions as properties exhibited by polar lipids are primarily influenced by their constituent unsaturated fatty acids.

Delta-6 -desaturation (the principal regulatory step in the biosynthesis of polyunsatuated fatty acids) takes

place when lineolyl-CoA is converted to gamma-lineolyl-CoA (Okayasu et al., 1979). The enzyme involved in this conversion, delta-6-desaturase has been purified from rat liver chromasomes and characterised by Okayasu et al. (1981). The enzyme was found to be a non-haeme protein containing one atom of iron per molecule. The single polypeptide has a molecular weight of ± 66 000 ± 1000, which is larger than delta-9-desaturase and consists of 15.1 ± 1.4 nmol of iron/mg protein. The amino acid composition was also determined. In rats desaturation is catalized by multienzyme complexes consisting of cytochrome b5 reductase, cytochrome b5 and the desaturase bound in the microsomal membranes. Omission of one of these components led to complete loss of activity. The authors postulated that the delta-6 and delta-9-desaturase were located differently in the complex. The delta-6-desaturase activity of a reconstituted system was extensively inhibited by potassium cyanide and p-chloromecuri-benzene sulphonate. Dithiothreitol and β-mercaptoethanol mildly inhibited the enzyme, while N-ethylmaleimide had little effect.

The fatty acids formed are rapidly esterified into complex lipids and in humans prostaglandins are formed by ring closure and oxygenation of the polyunsaturated fatty acids.

1.2.3 FATTY ACID DEGRADATION

Fatty acid degradation may take place via three mechanisms namely, alpha-oxidation, beta-oxidation and ω-oxidation. These oxidative pathways have been extensively studied in plants and animals, but almost no literature is available on these processes in fungi. It is, therefore, assumed that fatty acid oxidation takes place in fungi in a similar manner to that in higher eukaryotes, but a lot of research will have to be done to prove this assumption.

ω-oxidation catalised by microsomal enzymes of the 'oxygenase' type and its function is uncertain (Gurr and James, 1971). There are no reports available that this pathway is present in fungi. Alpha-oxidation is widely distributed in nature and involves oxidative decarboxylation of fatty acid in which CO2 and an acid containing one less carbon are produced. The exact mechanisms of the pathway are not well established and although there are several properties in common, the alpha-oxidation systems differ among the various tissues studied. In peanuts, the involvement of a perioxidase and an aldehyde dehydrogenase was indicated and in C. utilis, alpha-oxidation was suggested to be similar.

The beta-oxidation pathway (Fig. 3) of fatty acid degradation is the principle pathway of fatty acid catabolism. As mentioned earlier, the pathway is the

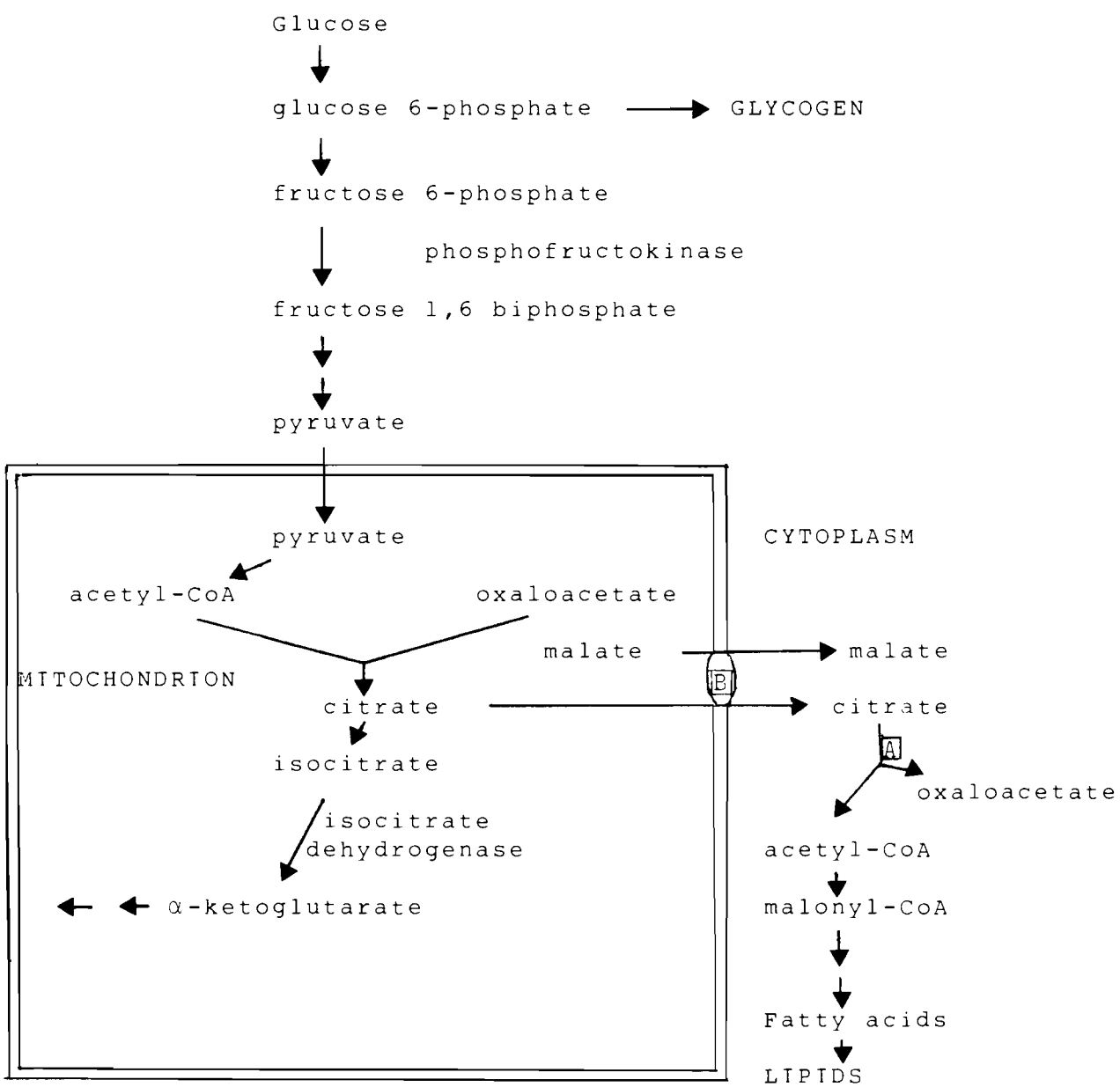
reversal of the fatty acid elongation process and the primary function of the pathway is to produce energy, in the form ATP, from fatty acids. Beta-oxidation takes place in the glycosomes or in the perisomes in fungi as compared to the cystolic location of the fatty acid synthesis.

1.2.4 BIOCHEMISTRY OF OLEAGINICITY

The biochemistry of oleaginicity has been reviewed by Ratledge (1982 & 1984). The reasons for oleaginicity in yeasts and fungi have until recently remained unresearched and the phenomenon seemingly regarded as a side issue in the main stream of lipid biochemistry. The key to oleaginicity in micro-organisms, with certian provisos, appears to reside in the possesion of a single enzyme, ATP:citrate lyase. This enzyme, which is normally thought to be present in plants and animals, had been previously reported in Rhodotorula gracilis and Penicillium spiculisporum and several species of Mortirella. Although the presence of this enzyme was correlated with lipid accumulation, the preliminary reports appear not to have been followed up. Subsequently, the enzyme has been found in a number of oleaginous yeast species and not found in a number of non-oleaginous yeasts. The mere presence of this enzyme, however, is not sufficient to cause lipid accumulation as it is still found to be active in carbon limited

oleaginous yeasts as well as having been detected in a non-oleaginous strain of R. gracilis. Other metabolic events must occur within the cell to provide the citrate needed as substrate for the enzyme. The major influence on metabolism is probably the build up of ATP and the depletion of AMP in cells whose growth is limited by the supply of nitrogen (or any other nutrient besides carbon). Glucose continues to be metabolised by such cells and provides a surplus of energy in the form of ATP, which provides the energy required for fatty acid biosynthesis. The concomitant absence of AMP (having all been converted to ATP) also affects intermediary metabolism by affecting isocitrate dehydrogenase which has a specific requirement for AMP (Fig 5). The isocitrate dehydrogenase activity will decline and isocitrate will accumulate and equilibrate with citrate. The citric acid will pass out of the mitochondrion and into the cytoplasm where it will be cleaved by ATP:citrate lyase to acetyl-CoA and oxaloacetate. The acetyl-CoA is converted into fatty acids as described previously. Oxaloacetate is converted into malate which becomes the counter anion for citrate transport. Malate is conveted back to oxaloacetate in the mitochondrion, thereby providing one of the two substrates for citrate synthesis. The citrate cleavage enzyme appears to be the first enzyme for lipid biosynthesis and in oleaginous yeasts and is probably the rate controlling step as well as being subject to feedback inhibition.

Fig.5: Pathway of glucose metabolism to lipid in oleaginous micro-organisms; [A] ATP citrate lyase; [B] citrate malate translocase system (Ratledge, 1984).



The apparent regulatory effect of fatty acyl-CoA esters on ATP:citrate lyase and acetyl-CoA carboxylase (1.2.2) may not only be a means whereby the cell regulates the amount of lipid it will synthesise, but may be a means of the cell preventing lipid biosynthesis when the cell is under conditions of starvation and the stored triacylglycerides are being utilized. The organism thus avoids two competing activities ie. synthesis and degradation. The first enzyme involved in the process, acetyl-CoA carboxylase, is found to be stimulated by the presence of citric acid.

The events depicted in Fig. 5 have been elucidated in several yeasts and confirmed in the oleaginous fungus Mucor circinelloides. These events are unique to oleaginous organisms.

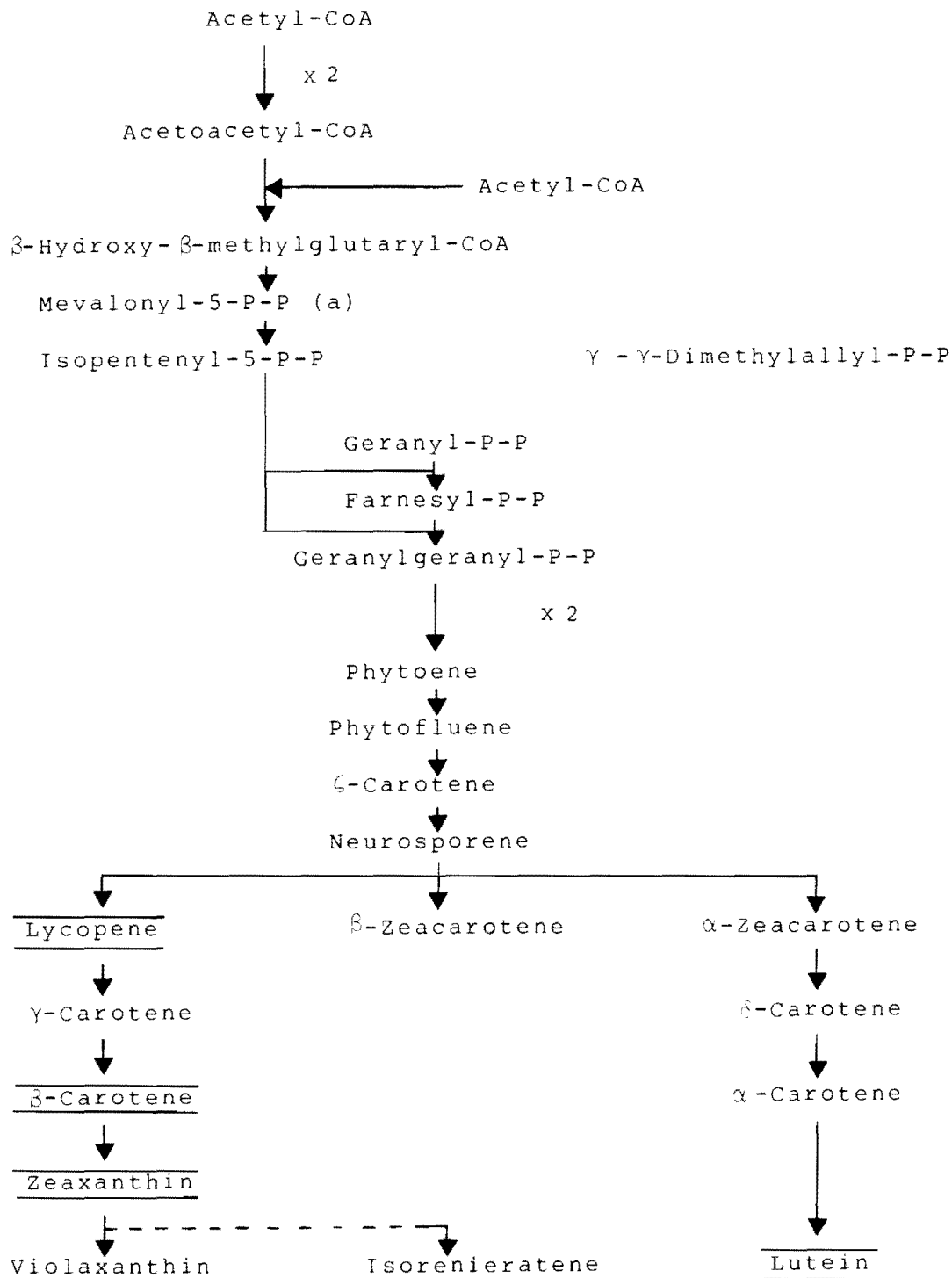
1.2.5 CAROTOGENESIS

Carotenoids form one of the most important families of natural pigments. They are liposoluble tetraterpenes, usually yellow to red in colour and are formed by the condensation of isoprenyl units. Only microorganisms and plants have the necessary biosynthetic machinery to synthesise a wide range of these substances. In practice, β-carotene and related carotenes are mainly found in fungi and algae, while xanthophylls occur almost exclusively in bacteria.

Of the Mucorales group of fungi, Blakeslea trispora, produces β-carotene at a sufficient rate to be used in an industrial fermentation. The pathway of carotenoid synthesis is outlined in Fig. 6. Lycopene is a deep red pigment which shows no provitamin A activity, unlike β-carotene. C. curcubitarum was found to produce β-carotene and it first appeared that there may be a link between β-carotene production and lipid production. This was investigated in chapter 3 and chapter 4. If a link was found it could be an easy and quick method to screen for mutants in lipid production.

In mixed cultures of (+) and (-) strains of C. curcubitarum, particularly in liquid medium, Barnett and Lilly (1956) found that the amount of β-carotene produced was greatly increased. This was apparently stimulated by hormone like substances excreted by the mycelium of the opposite sex. The β-carotene was concentrated in the suspenders and it was thought that it may have some function in sexual reproduction in the fungus.

Fig. 6: Pathway of carotenoid biosynthesis (Ninet and Renaut, 1979)



1.3 BIOTECHNOLOGY OF THE OILS AND FATS INDUSTRY

The world's demand for fat and oil in general is steadily increasing and the commercial potential of microbial oils has been assesed by Ratledge (1982). There is a limit to the amount of these materials that can be extracted from plant, animal and marine sources. At present 80 % of the oils and fats are derived from plant oilseeds which will always be governed by the climate. In recent years, therefore, there has been a resurgence of the concept of using micro-organisms as a source of oils. This interest has been futher stimulated by two factors: 1) technology for the continuous cultivation of organisms on an extremely large scale has been successfully developed by a number of companies interested in producing singe cell protein, and has led to the realization that micro-organisms can compete with cheap plant sources (provided the operation can be made large enough) and 2) the global price of oil is extremely volatile.

Biotechnology and the fats and oils industry has been reviewed by Ratledge (1984) and Rattray (1984). Biotechnology with micro-organisms can be seen as an alternative means of producing oils and fats, though the economics are against such processes being taken up for all but the most expensive oils and fats. The major impact of biotechnology is believed to be associated with the production of high value speciality products,

which would be applicable to the production of GLA. Improvements are being made to the quality and quantity of exsisting plant seed oils using agronomic practices and plant breeding programmes. A great deal of research is also being done on breeding better varieties of the evening primrose (Graham, 1984).

The introduction of new desirable strains of micro-organisms through genetic engineering has tremendous potential. Relatively very little work has been done on the genetics in fungi, even less on the Phycomyces fungi and no work has been published on the genetics of C. curcubitarum in particular. A great deal of ground work is therefore required before any degree of success can be achieved.

The aim of this thesis was to gain a basic understanding of the physiology and the environmental factors affecting the lipid and particularly GLA production in C. curcubitarum. Before any genetic programme can be implemented a suitable technique for screening mutants in lipid production must be developed and this problem has been addressed in chapter 2. The GLA production in the fungus was optimised in chapter 3 and in chapter 4 a few mutants in lipid, GLA and carotene production that were isolated are discussed.

CHAPTER TWO

EXTRACTION AND ANALYSIS

SUMMARY

A rapid method for the extraction and analysis of bacterial fatty acids, published by Hewlett-Packard (1984), was quantified and adapted for the analysis of mycelial lipid. The method has been termed the "HP method of extraction and analysis". The variance and the validilty of the method was examined. The variability of a method for the extraction and analysis of mycelial lipid developed by National Chemical Products (termed the "conventional method of extraction and analysis") was examined and the two methods compared. The HP method was found to be the method of choice.

The lipid extract from C. curcubitarum was fractionated and the GLA found to be mainly in the triacylglyceride fraction of the lipid.

SECTION A

METHOD OF EXTRACTION AND ANALYSIS

2.1 INTRODUCTION

This chapter has been divided into two sections.

Section A deals with the development of a quantitative method for the rapid extraction and analysis of the fatty acids in the mycelium of C. curcubitarum.

Section B consists of an analysis of the different fractions of lipid within the mycelium of C. curcubitarum. The aim was to determine in which fraction of the lipid the GLA was found.

The extraction and analysis of microbial lipid has been reveiwed by Ratledge (1982) and the following information, unless otherwise quoted, has been extracted from this reveiw.

"Nothing causes the newcomer to the field of the extracion and analysis of lipid more headaches than lipid extraction". There are two main pitfalls: 1) incomplete extraction and 2) the formation of free fatty acids during the extraction process.

Methods for the extraction of microbial lipid involve disrupting the cell either chemically or mechanically

and then refluxing with a combination of solvents for hours before the lipid extract can be esterified for analysis by gas chromatography.

Chemical disruption is achieved by treating with either acid or alkali. Although these methods will lead to some degradation of the lipid, this may not be a disadvantage if only the total lipid and/or the total fatty acid composition are required. These methods are not suitable if fractionation is subsequently planned.

Mechanical disruption of the cell can be achieved by ultrasonic treatment, shaking with glass beads in a Mickle or Braun vibrating shaker, passage through a French or Hughes press, freeze drying, oven drying, spray drying or incubation with enzymes. Enzymes are, however, not recommended as lipases and phospholipases are activated and cause degradation of some of the lipid fractions.

Co-extraction of a number of cell components often occurs. These may be removed if, for instance, the chloroform:methanol extract is shaken with a little water or 1 % (w/v) NaCl or KCl solutions [the "Folch" wash procedure (Folch et al., 1957)]. The crude lipid may be redissolved in solvent and the insoluble material removed by centrifuging or by filtration.

When lipid fractionation is performed, one of the biggest pitfalls is the reported presence of large

amounts of free fatty acids. These substanses are extremely toxic to the cell and their pressence indicates that lipases and phospholipases have been active after harvesting the micro-organism. These enzymes are actually activated in the presence of organic solvents and it has been suggested that freshly harvested cells should be placed in 80 % (v/v) ethanol at 80°C to inactivate the enzymes. This is then followed by cell disruption. Changes in the lipid composition can also occur if cells are allowed to stand for any length of time after harvesting or even held in the cold overnight.

The lipid yield of a micro-organism can vary markedly according to the solvent and method of extraction and analysis used.

A method for extracting the mycelial lipid was developed by researchers at National Chemical Products. The methyl esters of the extract were formed using a method described by Bannon et al. (1982) prior to analysis by gas-liquid chromatography. This extraction and analysis procedure has been termed "the conventional method of extraction and analysis" (2.2.2). The method is time consuming and not at all suitable for screening for mutants in lipid production.

The success of any genetic programme depends on a suitable screening technique and very few rapid methods of extraction and analysis have been developed. The aim

of this chapter was to find a rapid, quantitative method of extraction and analysis that would be suitable to screen for mutants in lipid production.

The first attempt to shorten the extraction method was made using chromatographic columns (2.2.1). The second attempt was more successful and was based on a method published by Moss et al. (1974) and modified by Hewkett-Packard (Miller, 1984) for the rapid extraction and analysis of bacterial fatty acids. The method has been termed the "HP method of extraction and analysis" (2.2.3).

2.2 MATERIALS AND METHODS

2.2.1 CHROMATOGRAPHIC METHOD OF EXTRACTION AND ANALYSIS

The chromatographic columns were poured with chromatographic grade silica gel (Merck) in hexane:acetone (1:1) and washed with acetone. Approximately 1g of the mycelium was vacuum dried and ground with 5g of silica gel using a pestle and mortar. The sample was transferred to the top of the column and the column was eluted with various solvents. The lipid extract was collected in a beaker at the bottom of the column.

The lipid was esterified for analysis by gas-liquid chromatography described in 2.2.2.2.

2.2.2 CONVENTIONAL METHOD OF EXTRACTION AND ANALYSIS

2.2.2.1 Extraction

The mycelium was harvested by either filtering under vacuum, using a Buchner funnel and Watmans No. 541 filter paper for liquid medium cultures, or peeling the mycelium off the agar medium plates. The mycelial lipid was then immediately extracted and analysed to prevent the formation of free fatty acids (2.1).

Samples of 10-15g of mycelium were homogenized with 200ml 1:1 chloroform-methanol (v/v) using an ultraturex. The homogenate was refluxed for an hour before the

mycelium was filtered from the lipid extract under vacuum. The filtrate was kept aside while the mycelium was resuspended in 200ml of the solvent and refluxed for another hour. The lipid extract from the second reflux was added to the first and the solvent removed using a rotary evaporator. The crude lipid extract was purified by resuspending it in 100ml hexane and allowing it to stand for an hour.

The purified lipid extract was dried with sodium sulphate and the extract was filtered before the solvent was evaporated off with a rotary evaporator. The lipid was then weighed.

The lipid extracts were stored in hexane, under nitrogen, at 4°C.

2.2.2.2 Esterification

Samples containing about 250mg of the purified lipid extact were transferred into 25ml volumetric flasks and approximately 50mg of pentadecanoic acid were added to each flask as an internal standard. The solvent was evaporated off in a water bath and 4 ml of 0.5N KOH in methanol was added to each sample. This was heated for 5-10 min to ensure that all the lipid was saponified. After cooling for 2-3 min, 5 ml of BF_3 in methanol (Merck) was added and the sample was heated for 2-3 min. A saturated salt solution was then added to float the

methyl esters into the neck of the flask.

A 10 % solution of the esters in hexane was used for analysis by gas-liquid chromatography. The column used was a Hewlett-Packard 20 M carbowax capillary column. The sample took 6 min to elute from the Hewlett-Packard 5880 chromatograph.

A calibration standard (for formula see appendix) was esterified in the same manner and used to calibrate the instrument and verify the instrument accuracy.

2.2.3 HEWLETT-PACKARD METHOD OF EXTRACTION AND ANALYSIS

The mycelium was harvested as described in 2.2.2.1. In the case of agar medium, three plates were harvested and the mycelial mats were placed on top of each other. The excess water was removed by pressing these mats between sheets of blotting paper. Cross-sections were then cut immediately prior to extraction and analysis.

Samples of 70-100mg were used. The analyses were performed in 5ml glass vials fitted with teflon lined caps and magnetic stirrers. A Reactitherm (Pierce®-heating and stirring module) was used as a heating block.

The mycelium and internal standard (pentadecanoic acid in methanol) was heated at 100°C for 30 min in 1.1ml of 1.2N NaOH and then cooled to room temperature. The

saponified extract was acidified with 0.5ml of 6N HCL. This was heated for 5 min at 85°C with 1ml of 12 % BCl3 in methanol (Merck). The esterified lipid was extracted by adding 1 ml of ether:hexane (1:1) and gently shaking the vial end over end for 3 min. The acidified bottom layer was discarded and after a base wash with 0.3N NaOH the top layer (lipid extract) was transferred to a vial that had been flushed with nitrogen using a gas-tight syringe. The sample was analysed by gas-liquid chromatography using the capillary column and chromatograph described in 2.2.2.2.

A calibration sample was esterified in the same manner.

For each batch of mycelium four samples were analysed and the average of the four results was used as the final answer.

2.2.4 DRY WEIGHT ANALYSES

Dry weight analyses were performed by heating at least 0.5g of the mycelium at 110°C for 2 h or until a steady mass was attained. The analyses were performed in duplicate as a degree of variation was found and the average of the two was used as the final answer.

2.2.5 FUNGAL STRAIN AND MEDIUM

The culture used was C. curcubitarum 12997 which was supplied by ATTC and the medium was Shaw's medium

which is listed in the appendix.

2.2.6 CHOICE OF UNITS

There is some discrepancy in the literature as to the definition of lipid yields.

The definitions used in this thesis are:

- mg/g fatty acid (F.A.) per 100g dry matter
- mg/g fatty acid (F.A.) per litre medium

The % GLA has been reported as the % found in the total F.A. produced and not as the % in the total lipid in the mycelium.

2.3 RESULTS AND DISCUSSION

2.3.1 EXTRACTION METHOD USING A CHROMATOGRAPHIC COLUMN

The lipid in the mycelium was extracted and analysed as described in 2.2.1. The solvents used to elute the column were:

1) 1:1 hexane:acetone
2) 1:1 hexane:acetone followed by methanol
3) 2:1 chloroform:methanol

A sample of the mycelium was extracted by the conventional method (2.2.2) as a control.

The results (Table 2.1) show an approximately 50 % decrease in the amount of GLA and fatty acids extracted by the column method as compared to the control. This seems to indicate that the remaining fatty acids were either retained on the packing material or the lipid was insufficiently extracted from the mycelium. Possible investigation into other packing materials could be considered.

The fatty acids took approximately 30 min to elute from the column and the solvent was then evaporated off using a rotary evaporator and weighed.

There was considerable time and effort saving using the column method of extraction as compared to the conventional method. However, far less lipid was

Table 2.1: GLA and fatty acid yields obtained using a chromatographic column for extraction.

The GLA and the fatty acid from C. curcubitarum were extracted and analysed by the chromatographic column method described in 2.2.1 and compared to a control where the analysis was performed using the conventional method described in 2.2.2.

SAMPLE	% GLA IN F.A.	GLA mg/100g dry matter	FATTY ACID mg/100g dry matter
CONTROL	15.2	1171.3	7.72
1)	15.8	624.8	3.95
2)	15.8	660.1	4.17
3)	15.1	668.9	4.43

extracted in this manner and a large number of chromatographic columns would have to be set up in order to process a large number of samples. Once the lipid has been extracted, the esterification must still be performed prior to analysis. For the esterification of a large number of samples, the procedure is too time consuming to be viable as a method for screening mutants in lipid production.

2.3.2 CONVENTIONAL METHOD OF EXTRACTION AND ANALYSIS

Agar and liquid medium cultures were innoculated with C. curcubitarum and incubated under identical conditions. The lipid was extracted and analysed by the conventional method (2.2.2) and the results were compared in order to check the variability of the method.

In the case of liquid medium, three 2L Ehrlenmeyer flasks containing 600 ml of Shaw's medium were innoculated with a total of 9.75×10^5 spores. The cultures were incubated at RT, in a Gallenkamp orbital shaker at 80 r.p.m., for four days.

Nine Shaw's agar medium plates (20ml/plate) were innoculated by placing a piece of mycelium, from a four day old culture grown on GYE/Novobiacin plates, in the centre of each plate. The plates were incubated at RT, for four days, under conditions of alternating light and darkness. Three plates were harvested for each analysis.

The results are tabulated in Table 2.2A and B.

Statistically, the variability of the results obtained from mycelia grown in liquid and on agar media was the same. The only significant difference (F test for equality of standard deviations at the 5 % level) was the % GLA in the fatty acids, where the agar medium results showed less variability. The GLA/l medium

Table 2.2A: Summary statistics of the conventional method in the extraction and analysis of mycelium grown in Shaw's liquid medium.

SAMPLE NO.	DRY MATTER g/l	% GLA IN F.A.	GLA mg/100g dry mat.	GLA mg/l medium	FATTY ACID g/100g dry matter	F.A. g/l medium
1	4.92	14.1	2385.0	117.3	16.9	0.83
2	5.48	17.2	2240.0	122.8	13.0	0.71
3	6.13	10.9	1753.0	107.5	16.1	0.98
MEAN	5.51	14.1	2126.0	115.9	15.3	0.84
SD	0.61	3.2	331.1	7.8	2.1	0.14
RSD (%)	11.1	22.7	15.6	6.7	13.7	16.7

Table 2.2B: Summary statistics of the conventional method in the extraction and analysis of mycelium grown on Shaw's agar medium.

SAMPLE NO.	DRY MATTER g/l	% GLA IN F.A.	GLA mg/100g dry mat.	GLA mg/l medium	FATTY ACID g/100g dry matter	F.A. g/l medium
1	15.96	18.4	3133.7	500.1	17.0	2.72
2	14.80	18.1	3967.1	587.1	21.9	3.24
3	13.40	18.5	3827.0	512.8	20.8	2.78
MEAN	14.72	18.3	3642.6	533.3	19.9	2.91
SD	1.28	0.2	446.3	47.0	2.6	0.28
RSD (%)	8.7	1.1	12.3	8.8	13.1	9.62

was significantly less variable in the agar medium results at the 10 % level, but just short of being significant at the 5 % level.

2.3.3. HEWLETT-PACKARD METHOD OF EXTRACTION AND ANALYSIS

The standard calibration mixture was esterified by the HP method described in 2.2.3 and the chromatograph calibrated with the sample. The esterified calibration was then injected several times and the recoveries were well within the 5 % margin.

The reproducibility of the calibration was checked by esterifying and analysing a number of samples of the calibration mixture. Again all the results were well within the accepted 5 % margin.

Samples of 50mg of oil extracted from C. curcubitarum were spiked with 2, 5, 10 and 15mg amounts of GLA and analysed to check the recoveries. The recoveries varied from 108.1 % to 92.8 %, which is highly acceptable when working with such small quantities of oil.

Esterified fatty acid samples prepared by the HP method were found to be stable for up to two weeks when kept in ether:hexane (1:1), at 4°C , under nitrogen.

2.3.3.1 VARIABILITY OF THE HP METHOD OF EXTRACTION AND ANALYSIS

The variablility of the HP method of extraction and analysis was examined as in 2.3.2., using mycelium grown in both liquid and agar media. The liquid medium cultures were grown in 200ml of Shaw's medium in 500ml Ehrlenmeyer flasks and innoculated with a total of

Table 2.3A: Summary statistics of the HP method in the extraction and analysis of mycelium grown in Shaw's liquid medium.

SAMPLE NO.	DRY MATTER g/l	% GLA IN F.A.	GLA mg/100g dry mat.	GLA mg/l medium	FATTY ACID g/100g dry matter	F.A. g/l medium
1	5.10	6.4	1048.3	53.47	16.3	0.83
2	5.35	6.1	933.6	49.95	15.4	0.82
3	4.95	7.0	1178.6	58.34	16.8	0.83
MEAN	5.13	6.5	1053.5	53.92	16.2	0.83
SD	0.20	0.5	122.6	4.21	0.71	0.01
RSD (%)	3.9	7.7	11.6	7.8	4.4	1.2

Table 2.3B: Summary statistics of the HP method in the extraction and analysis of mycelium grown on Shaw's agar medium.

SAMPLE NO.	DRY MATTER g/l	% GLA IN F.A.	GLA mg/100g dry mat.	GLA mg/l medium	FATTY ACID g/100g dry matter	F.A. g/l medium
1	12.07	16.5	4553.4	549.6	27.7	3.34
2	12.70	16.7	4271.5	542.5	25.7	3.26
3	11.40	15.6	4685.4	534.1	30.2	3.44
MEAN	12.06	16.3	4503.4	542.1	27.9	3.35
SD	0.65	0.59	211.4	7.7	2.3	0.09
RSD (%)	5.4	3.6	4.7	1.4	8.2	2.7

Table 2.3C: Results obtained using the HP method in the extraction and analysis of mycelium grown in Shaw's liquid medium.

SAMPLE NO.	DRY MATTER g/l	% GLA IN F.A.	GLA mg/100g dry mat.	GLA mg/l medium	FATTY ACID g/100g dry matter	F.A. g/l medium
A-1	5.10	6.2	962.3	49.08	15.4	0.79
A-2	5.10	7.1	1185.2	60.45	16.7	0.85
A-3	5.10	6.2	925.1	47.18	14.8	0.76
A-4	5.10	6.2	1120.7	57.16	18.1	0.92
B-1	5.35	5.9	904.2	48.38	15.4	0.82
B-2	5.35	5.8	1002.4	53.63	17.1	0.92
B-3	5.35	6.3	833.6	44.60	13.3	0.71
B-4	5.35	6.3	994.1	53.18	15.6	0.84
C-1	4.95	6.3	1096.4	54.27	17.4	0.86
C-2	4.95	7.2	1214.1	60.10	16.7	0.83
C-3	4.95	7.2	1193.4	59.07	16.6	0.82
C-4	4.95	7.3	1210.3	59.91	16.6	0.82

Table 2.3D: Results obtained using the HP method in the extraction and analysis of mycelium grown on Shaw's agar medium.

SAMPLE NO.	DRY MATTER g/l	% GLA IN F.A.	GLA mg/100g dry mat.	GLA mg/l medium	FATTY ACID g/100g dry matter	F.A. g/l medium
A-1	12.07	16.3	4485.9	541.5	27.6	3.33
A-2	12.07	16.5	4551.9	549.4	27.7	3.34
A-3	12.07	16.8	4445.3	536.6	26.5	3.21
A-4	12.07	16.4	4730.4	571.0	28.9	3.49
B-1	12.70	17.5	3919.4	497.8	22.4	2.85
B-2	12.70	15.7	4047.7	514.1	25.8	3.28
B-3	12.70	16.5	4537.9	576.3	27.5	3.49
B-4	12.70	17.0	4581.0	581.8	27.0	3.43
C-1	11.40	14.9	4155.2	473.7	28.0	3.19
C-2	11.40	15.6	4870.0	555.2	31.3	3.57
C-3	11.40	16.2	4755.4	542.1	29.4	3.35
C-4	11.40	15.5	4961.1	565.6	31.9	3.64

Table 2.3E: Statistical significant differences between mutants of C. curcubitarum and the WT using the HP method of extraction and analysis.

The amount, (X), by which a mutant should differ from the WT, to be statistically significant at the 5 % level, was calculated using the data in Table 2.3D and the T table; where

$$X = \frac{\text{T value} \times \sqrt{2} \times \delta}{\sqrt{n}}$$

n = number of samples analysed.

δ = SD (% GLA) = 0.719

δ = SD (GLA/l medium) = 32.779

δ = SD (F.A./l medium) = 0.209

The T value is multiplied by the $\sqrt{2}$ to take account of the variability of the WT as well as the variability of the mutant, (since the variance of a difference is equal to the sum of the individual differences).

NO. OF ANALYSES PER SAMPLE	T VALUE	AMOUNT BY WHICH THE MUTANT SHOULD DIFFER TO BE SIGNIFICANT (X)		
		% GLA	GLA /l medium	F.A./l medium
2	12.706	9.1	416.4	2.7
3	4.303	2.6	116.2	0.7
4	3.182	1.6	73.0	0.5
5	2.776	1.3	57.9	0.4
12	2.201	0.6	28.9	0.2

9.75×10^5 spores.

The mycelium was harvested after incubation for four days and the lipid extracted and analysed by the HP method described in 2.2.3. The results are tabulated in Table 2.3A and B. Table 2.3C and D show the results of the individual analyses of the mycelium grown in liquid and on agar medium respectively. (The average of each four of these analyses are shown in Table 2.3A and B).

Statistically, the variability of the results obtained from mycelium grown in the liquid and on agar medium was the same. The only significant difference (F test for the equality of standard deviations at the 5 % level) was the F.A./l medium where the liquid medium results showed less variability. It was decided to use Shaw's agar medium because solid medium is a more convenient medium for genetic studies.

The F-Ratio (which takes into account both the "within group" variance and the "between group" variance) for both the liquid and agar medium results for % GLA in the fatty acids and the mg GLA/l medium was calculated. The ratio was found to be well below the critical value of 9.55 for the set of conditions. i.e. It cannot be stated, at the 5 % level of significance, that the means of the samples analysed were statically different and therefore it is assumed that they originate from the same population (Table 2.3C and D).

Assuming that the standard deviation (SD) of the mutants from their mean is the same as the SD of the wild-type (WT) from its mean, the number of SD's a mutant must differ from the WT, at the 5 % level of significance, can be calculated (Table 2.3E).

A system for screening for mutants in lipid production using the HP method of extraction and analysis was thus developed.

The mutants were grown on Shaw's agar medium and the WT was used as a control. For the initial screening dry weight analyses were not performed and the mutants were selected by either an increase or a decrease in the amount of GLA or F.A./l medium or % GLA in the fatty acids. Three samples of the mutant were analysed. If the mutant differed by the significant figure (eg. Table 2.3E), the mutant was set aside for further investigation where dry weight analyses were also performed and four samples of each mutant were analysed. The carC2 series of mutants were screened using this system (4.3.4).

The HP method of extraction and analysis could not be used if total lipid produced by the fungus or carotene levels in the mycelium are to be determined.

2.3.3.2 HP METHOD USING INCREASING AMOUNTS OF MYCELIUM

Increasing amounts of mycelium, from 50mg to 121mg, were weighed and the lipid extracted and analysed by the HP method described in 2.2.3. The amount of GLA extracted was plotted against the amount of mycelium analysed (Fig. 2.4A and B).

Mycelium grown on both agar and in liquid medium was used and in both cases a straight line was obtained.

A linear regression on the results obtained when the mycelium was grown on agar medium (Fig. 2.4A) shows that the regression line can be represented by the following equation:

$$Y = 0.01X + 0.1$$

The product momentum co-efficient, r, (i.e. the correlation between X and Y), equals 0.997 and that for a perfect direct relationship is 1.

Similarly, a linear regression on the liquid medium results show that the regression line in Figure 2.4B can be represented by the equation:

$$Y = 0.01X + 0.05$$

The product momentum co-efficient, r, equals 0.996 in this case.

There is, therfore, a direct relationship between the no. of mg of GLA extracted and the amount of mycelium analysed by the HP method of extraction and analysis.

Fig. 2.4: The relationship between the amounts of C. curcubitarum mycelium extracted and the amounts of GLA obtained when using the HP method of extraction and analysis.

The amount of wet mycelium used for extraction (mg) from an agar medium culture (Fig. 2.4A) and a liquid medium culture (Fig. 2.4B) was plotted on X and the amount of GLA (mg) extracted was plotted on Y.

Fig. 2.4A:

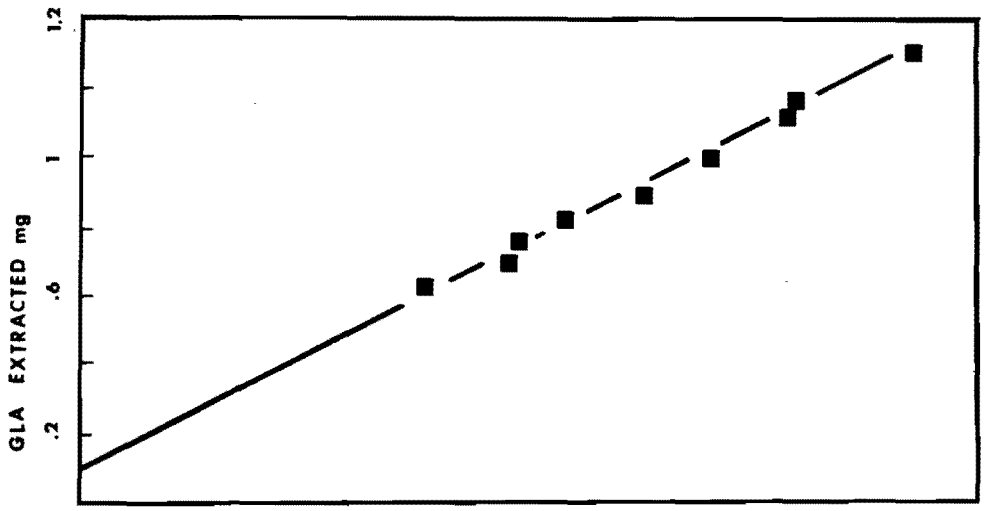
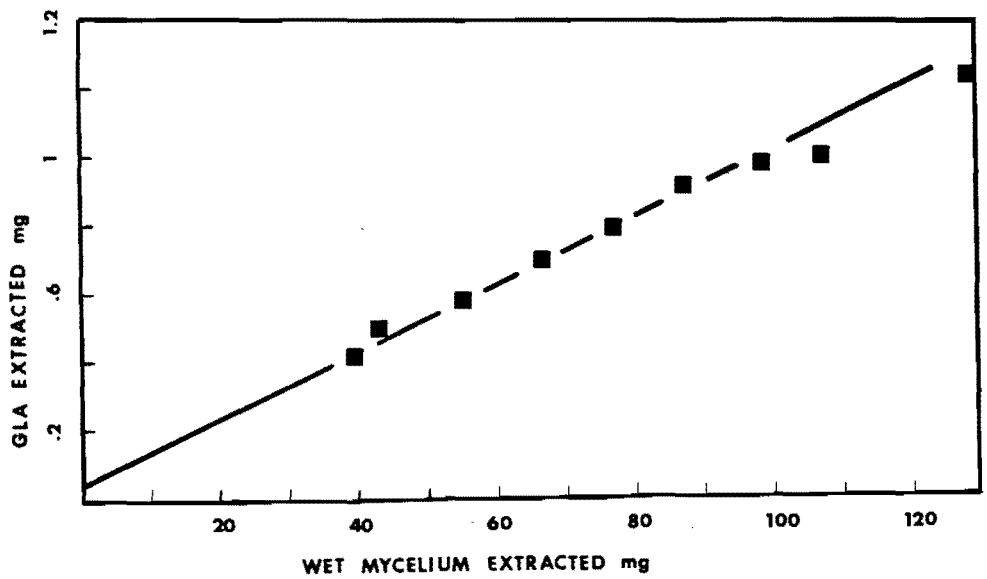


Fig. 2.4B:



2.3.4 COMPARING THE HP TO THE CONVENTIONAL METHOD OF EXTRACTION AND ANALYSIS

In order to compare the HP method (2.2.3) to the conventional (2.2.2) method of extraction and analysis, mycelium, from a four day old batch culture grown in a Chemap batch fermentor, was extracted and analysed by both methods and the results compared. In addition, mycelium that had already been extracted by the conventional method was extracted by the HP method to see if there was any fatty acid left in the mycelium.

The results obtained for GLA and total fatty acids were very much higher for the HP extraction than for the conventional extraction (Table 2.5A). Once the conventional extraction had been completed, an HP extraction on the mycelium showed that there was a substantial amount of lipid left in the mycelium.

Assuming that the HP method extracts all the lipid available, it was found that only 57.7 % of the GLA and 50.9 % of the total fatty acids were extracted by the conventional method (Table 2.5A). All the results in chapter 3 that were obtained using the conventional method of analysis, therefore, could be doubled to reflect the true quantity of fatty acids produced in the mycelium.

The % GLA found in the fatty acids was higher for the conventional method. This could be attributed to one or

Table 2.5A: The influence of the HP method and the conventional method of extraction and analysis on the GLA and fatty acid yields of C. curcubitarum.

EXTRACTION METHOD	DRY MATTER g	% GLA in F.A.	GLA mg/100g dry mat.	GLA in biomass	F.A. g/100g dry mat.	F.A. in biomass
HP	74.34	13.1	3086.5	2294.5	23.6	17.54
CONVEN.	74.34	17.0	1939.9	1442.1	11.6	8.62
HP after conven.	56.52	12.7	1867.4	1055.5	14.7	8.31

Table 2.5B: The influence of the method of extraction and analysis on the degree of saturation and unsaturation in the fatty acids produced by C. curcubitarum.

FATTY ACIDS	CONVENTIONAL METHOD % w/w	HP METHOD % w/w
MYRISTIC (14:0)	1.7	1.1
PALMITIC (16:0)	28.6	29.6
PALMITOLEIC (16:0)	3.4	2.6
STEARIC (18:0)	5.4	8.3
OLEIC (18:1)	25.9	27.8
LINOLEIC (18:2)	18.0	17.5
GLA (18:3)	17.0	13.1
% UNSAT.	64.3	61.0
% SAT.	35.7	39.0

a combination of several reasons. The conventional method may have been extracting less of the other fatty acids or selectively extracting GLA. Examination of the response factors obtained when calibrating the chromatograph indicated that the amount of stearic acid reported was consistently high and this may be the reason that the % GLA was lower. On the other hand, the total unsaturation is 3.3 % lower in the HP method of extraction and analysis (Table 2.5B), which could indicate that the harsh physical conditions of the method actually destroys the more reactive unsaturated fatty acid, GLA.

There is, however, a direct relationship between the amount of of GLA extracted and the amount of mycelium analysed (2.3.3.2), which indicates that the method is extracting a constant amount of GLA and since all the results are relative, any discrepaancy would not be significant.

The variability of the results obtained using the conventional and the HP method of extraction and analysis was the same (F test for the equality of standard deviations at the 5 % significance level on results tabulated in Tables 2.2A & B and Tables 2.3A & B). The observed variability was, however, less in the HP method than the observed variability of the conventional method (except in the results obtained using agar medium where the observed variabilily was

less for the conventional method of extraction and analysis; Table 2.2B and 2.3B).

There is considerable time and effort saving using the HP method as compared to the conventional method of extraction and analysis. Nine samples may be analysed within an hour using the HP method, whereas six samples take up to two days to analyse using the conventional method of extraction and analysis. The HP method is, therefore, recommended and was the method of choice.

SECTION B

FRACTIONATION OF THE LIPID OF *C. curcubitarum*.

2.5 INTRODUCTION

The aim of this section was to determine in which fraction of the lipid of *C. curcubitarum* the GLA is found. The research was done for two reasons:
1) To determine whether an increase in the lipid globules in the mycelium would indicate an increase in GLA content and thus provide a means by which mutants in lipid production could be screened, using a stain such as Sudan Black or Nile Blue (4.3.1).
2) To try and gain some insight into the actual function of GLA in the mycelium of the fungus.

The lipid content of the mycelium can be fractionated by using enzymes to digest the cell wall or by using silicic acid absorption chromatography (Hirsh and Ahrens, 1958).

By using enzymes to dissolve the cell wall, the cell contents could be released from the protoplasts and the two fractions, (the cell wall and membrane and the cytoplasm), separated by centrifugation. Each fraction could then be analysed for it's GLA content. Protoplasts could also be used at a later stage for protoplast fusion.

The ability and efficiency of cells to convert to protoplasts, using enzymes, is dependent on the type and strain of the organism and on it's physiological state. It is, therefore, necessary to design the method to fit the particular organism. Yeasts are the only group of fungi in which conversion of cells into spherical osmotically labile bodies has been achieved by selective removal of the cell wall. Far less quantitative conversion into protoplasts has been achieved with filamentous fungi (Villanueva and Acha, 1971).

Several workers have caried out simple separations of lipids into neutral and polar fractions (Ratledge, 1982).

White and Powel (1966) used thin layer chromatography to determine that the triacylglycerol was the most abundant component of the fungal lipid of C. curcubitarum. Phospholipids and free sterol were also present in substantial proportions whereas, only traces of free fatty acids and sterol ester / hydrocarbon mixture were found. The predominant fatty acid in the phospholipids was linoleic acid (39 %) and palmitic acid (42 %) was the predominant free fatty acid. The partition of the individual fatty acids among the different lipid fractions was distinctive. The overall unsaturation was reported to be much higher in the phospholipids (73 %) than in the triacylglycerol fraction of the lipid (56 %).

In the related fungi, Cunninghamella elegans and Cunninghamella echinulata, the triacylglycerols constituted 85 % of the total lipid (Ratledge, 1982).

Shaw (1965) examined a few fungi belonging to the Phycomycetes, including C. curcubitarum, and reported that the proportion of GLA in the triacylglycerol fraction was very similar to that in the total extractable lipid. He postulated that it was, therefore, likely that the GLA was mainly present in the storage lipids in the Phycomycetes. Shaw (1966a) found that GLA was divided in equal portions between the compound and the neutral lipids of Cunninghamella blakesleeanus. Simple fractionation of the lipid revealed no differential distribution of the fatty acids in the various fractions of the lipid.

2.6 MATERIALS AND METHODS

2.6.1 ENZYMATIC DIGESTION OF THE CELL WALL

The mycelium was harvested after two days of incubation in Shaw's medium as protoplast formation is reported to be more succesful using young hyphae (Villanueva and Acha, 1971) and was then washed with buffer solution. Samples of 10mg dry weight /ml buffer were used in the experiments. The mycelium was incubated with the enzyme in 10 ml of buffer in a petri dish, secured in an orbital shaker, at RT, 30°C and 35°C.

The following enzymes and combinations thereof, in concentrations ranging from 0.1 - 5.0 %, were used: Snail digestive juices (Merc), Zymolase , Chitinase, Cellulase , Driselase (Fluka) and Novozyme (Novo Industries).

The following buffer solutions were used: 0.1 M Phosphate buffer and 0.8 M mannitol at pH 5.8 and at pH 6.8; 0.1 M phosphate buffer and 0.5M mannitol at pH 5.0 and 6.0: 10mM immidazole, 10mM MgCl3 and 1.2M sorbitol at pH 7.2.

Protoplast formation was monitored at 0.5, 1, 2, 3, 6, 24 and 48 h.

2.6.2 SILICIC ACID COLUMN CHROMATOGRAPHY

The culture used was C. curcubitarum 12997 which was obtained from ATTC. The medium used for incubation was Shaw's liquid medium which is listed in the appendix.

The lipid from was extracted from a four day old culture by the conventional method (2.2.2.1). A sample of the lipid extract was esterified (2.2.2.2) and analysed by gas-liquid chromatography as a control.

The lipid was separated on a silicic acid column as described by Kemp et al. (1984). The column was poured using 38g of silicic acid (lipid chromatography grade, Sigma) in CHCl3:MeOH (1:1, v/v). The column (30.5cm x 2.2cm) was washed with acetone and then CHCl3. The lipid extract (730mg) was applied to the top of the column in CHCl3. The column was then successively eluted with four column volumes (320ml) of CHCl3, acetone and CHCl3:MeOH (1:1, v/v) and the different fractions were collected in beakers at the bottom of the column.

The neutral lipids and the free fatty acids were eluted with the CHCl3, the glycolipids with the acetone and the phospholipids with the CHCl3:MeOH (1:1, v/v).

The fractions collected were esterified and analysed as described in section 2.2.2.2.

2.7 RESULTS AND DISCUSSION

2.7.1 ENZYMATIC DIGESTION OF THE CELL WALL

Using various combinations of enzymes and buffer solutions, weakening and breakages in the wall were noted after 24 h, but protoplast formation was not observed. The most effective enzyme combination was : Novozyme (100mg) and Snail digestive juices (1ml) in 5ml phosphate buffer at pH 5 and incubated for 24 h at 37°C.

Even after 48h there was no effective digestion of the cell wall, and as rapid digestion would be required to avoid changes in the fatty acid composition during incubation, it was decided to use silicic acid chromatography as an alternative means of determining how the GLA is fractionated in the lipid of *C. curcubitarum*.

2.7.2 SILICIC ACID COLUMN CHROMATOGRAPHY

The lipid from C. curcubitarum was extracted and analysed as described in 2.6.2.

The neutral fraction comprised 83 % of the sample applied to the top of the column (606.21 mg). The glycolipids comprised 14.8 % of the sample (107.7 mg) and the phospholipids comprised 1.1 % of the sample (8.0 mg).

The breakdown of the individual fatty acids into the different fractions of lipid eluted from the column is tabulated in Table 2.6A. Of the total GLA present, 95.7 % was found in the neutral and free fatty acid fraction of the extract, 1.5 % of the GLA was found in the glycolipids and 2.3 % in the phospholipids. This supports Shaw's postulation that the GLA is present mainly in the storage lipids (Shaw, 1965; section 2.5). An increase in the lipid globules in the mycelium could, therefore, be a good indication of increased GLA production in C. curcubitarum. The GLA was found in equal proportions in the neutral and glycolipids (21.6 % and 21.9 % respectively). The proportion of GLA in the phospholipids was slightly higher (26.4 %). The % unsaturation in the phospholipid fraction was higher than that in the neutral fraction, but not in the same magnitude as that reported by White et al. (1962; section 2.5).

Of the 730.08 mg applied to the top of the column, 1.2 %

was not eluted. This fraction could be the polar pigment that remained at the top of the column or unusual or branched chain fatty acids that may be present in the extract.

The neutral fraction was comprised of 45.4 % of unknown compounds. Carotene eluted with this fraction and comprised part of this unknown fraction. Sterols and sterol esters also elute with the neutral fraction of the lipid (Ratledge, 1982). No unknown compounds were found in the phospholipid fraction and 95.24 % of the glycolipids were unknown (most likely non-conjugated unknown compounds).

The % of the individual fatty acids eluted from the column correlated well with the control (Table 2.6B).

Table 2.6A: Fatty acid composition of the various fractions of lipid of C. curcubitarum.

The lipid extracted from a four day old culture of C. curcubitarum was fractionated using silicic acid adsorption chromatography.

FATTY ACID	NEUTRAL LIPIDS mg	GLYCOLIPIDS mg	PHOSPHOLIPIDS mg	TOTAL mg
MYRISTIC (14:0)	2.94	-	-	2.94
PALMITIC (16:0)	90.29	1.18	1.93	93.34
PALMITOLEIC (16:1)	12.11	-	-	12.11
STEARIC (18:0)	11.20	0.39	-	11.59
OLEIC (18:1)	89.79	1.77	2.41	93.97
LINOLEIC (18:2)	53.33	0.67	1.59	55.59
GLA (18:3)	71.35	1.13	2.12	74.59
TOTAL	331.02	5.14	8.04	344.18
UNKNOWNS	276.19	102.86	-	377.51
UNKNOWNS %	45.40	95.24	-	52.30
UNSAT. %	68.4	69.5	76.0	-
SAT. %	31.6	30.5	24.0	-

Table 2.6B: The % (w/w) analysis of the results obtained when fractionating the lipid of C. curcubitarum using silicic acid adsorption chromatography as compared to the control.

FATTY ACID	SAMPLE % w/w	CONTROL % w/w
MYRISTIC (14:0)	0.41	0.46
PALMITIC (16:0)	12.94	13.41
PALMITOLEIC (16:1)	1.68	1.96
STEARIC (18:0)	1.61	1.37
OLEIC (18:1)	13.02	11.49
LINOLEIC (18:2)	7.70	7.81
GLA (18:3)	10.33	11.84
TOTAL	47.70	48.34
UNKNOWNS	52.30	51.66

2.8 GENERAL CONCULSION

The HP method of lipid extraction and analysis provides a rapid and quantitative method suitable for the screening of mutants in GLA and lipid production. The extraction is highly efficient, requires minimum handling and very little equipment is used.

The limitation of the system is that the Reactitherm used is designed to hold only 9 samples. Many more samples could be analysed if several heating blocks were used and the samples prepared could be analysed overnight using an automatic sampler.

The GLA was found to be mainly in the neutral fraction of the lipid of C. curcubitarum. A staining technique that would show an increase in the lipid globules in the mycelium could, therefore, be used to screen for mutants in GLA production.

As the GLA is present in the triacylgylcerol fraction of the lipid and thus in the storage lipids of C. curcubitarum, it can be postulated that the main function of GLA in the mycelium is that of a reserve energy source. Since the % GLA is slightly higher in the phospholipids, the fatty acid could also play a role in the formation of the cell membrane in the fungus.

Protoplasts could not be produced in C. curcubitarum and therefore protoplast fusion cannot be used as a possible

genetic system for this fungus.

CHAPTER THREE

FACTORS AFFECTING GLA AND LIPID PRODUCTION BY *C. curcubitarum*

SUMMARY

The factors affecting GLA and lipid production by *C. curcubitarum* were investigated. The age of the culture, the C:N ratio in the medium, temperature, pH and aeration markedly affected the lipid production. Lipid accumulation during growth was followed by a phase of lipid utilization when the carbon source was limiting. The optimum conditions for GLA and lipid production were : a C:N ratio of between 20:1 and 30:1; an incubation temperature of 30°C; a pH of between 5.0 and 6.0; and maximum aeration to the medium during incubation. Carotogenesis and lipid production were not linked during the fermentation. Shaw's medium was shown to be optimum for GLA production.

3.1 INTRODUCTION

The factors affecting fat production by micro-organisms have been reviewed by Weete (1974a), Wassef (1978) and Ratledge (1982) and the following information, unless otherwise quoted, has been extracted from these reviews.

The amount of lipid produced by any given species of fungus depends to a great extent on the developmental stage of growth (i.e. age of the culture) and on the chemical and physical properties of the environment.

Wide fluctuations in the total lipid content in the order Mucorales are found during fat accumulation and depletion, even though the relative fatty acid composition varies little (Shaw, 1966a). In both C. curcubitarum and Rhizopus arrhizus however, a fall in the oleic acid and an increase in the linoleic acid content with age was found. (White et al, 1962; Shaw, 1966a). Moon and Hammond (1978), on the other hand, found that the relative fatty acid content did vary in aging cultures of Candida curvata and Trichosporon cutaneum.

The environmental parameters that influence the growth and lipid content are the carbon and nitrogen source, the carbon to nitrogen ratio (C:N ratio) in the medium, temperature, pH, aeration and vitamins. The extent to which these parameters affect lipid production varies from species to species and a medium formulation that

would be universally applicable does not exist. Even the closely related fungi R. arrhizus and Cuninghamella blakesleaanus differ in their requirements (Shaw 1966b) and as much as 100 % variation can be obtained from growing a fungus on different media.

The maximum theoretical conversion of substrate to lipid is only 33 % as there is a high loss of CO2 during the process and lipid is chemically much more reduced than carbohydrate and consequently contains far fewer oxygen atoms than the starting carbohydrate. This theoretical efficiency of lipid production can never be attained as it assumes that there is no diversion of glucose to other cell materials. In practice, efficiencies of fat formation (expressed in terms of the fat-coefficient, see 3.2.6) rarely exceed 22g of lipid from 100g carbohydrate substrate.

3.1.1 GROWTH

There are three patterns of lipid accumulation in oleaginous micro-organisms.

The first pattern of fat accumulation, which occurs in most oleaginous organisms growing in batch culture has two stages. The first stage is the proliferation of the cells growing at their maximum rate and this continues until a nutrient other than carbon, usually nitrogen, becomes depleted. The organism can then no longer

synthesise protein, DNA, and RNA but continues to take up carbon from the medium which is converted into lipid. There is no increase in the actual rate of lipid synthesis, the lipid accumulates because other processes cease.

The second pattern, where the organism seems to be constitutive for fat accumulation or always has a high fat content, has only been found in the yeast Cryptococcus tericolus.

The third pattern, where fat accumulation occurs during the phase of active growth, has been found in Phycomyces blakesleeanus and R. arrhizus which both belong to the Phycomycete group of fungi. These fungi rapidly synthesise and accumulate lipid during logarithmic growth and this is followed by a utilization phase during reproductive and stationary growth. Garton et al. (1951) found that maximum growth and lipid production occurs within 5 to 6 days of innoculation in P. blakesleeanus. C. curcubitarum is reported to accumulate lipid throughout a twelve day period of incubation (White and Powell, 1966).

The growth cycle and the rate of growth have a considerable influence over the composition of the lipid. These, in turn, are markedly influenced by the composition of the media and the environmental conditions during growth.

3.3.1.1 CAROTENE PRODUCTION

While investigating the growth pattern and its effect on fat production it was decided to examine carotene production in C. curcubitarum. It was hoped that if there was a link between lipid or GLA production, the pigment could be used as a method to screen for mutants in lipid production.

The predominant pigment found in P. blakesleeanus is β-carotene (Garton et al., 1951) and it was assumed that this was the case in C. curcubitarum as they both belong to the Phycomycete group of fungi.

Garton et al.(1951) found that even though the maximum growth and lipid production by P. blakesleeanus occurred within 5-6 days, maximum β-carotene production occurred a few days later. This would suggest that carotogenesis and fat production are not linked. However, the selection of β-carotene mutants may be advantageous to lipid production if the mutation occurs early in the β-carotene pathway, as AcetylCoA is common to both metabolic pathways (1.2.2 & 1.2.5).

3.1.2 THE CARBON AND NITROGEN SOURCE IN THE MEDIUM

The carbon and nitrogen source in the medium have the most pronounced effect on fungal lipid production. Farag et al.(1983) investigated the effects of the

various carbon and nitrogen sources using the fungi Tolyposporium ebrenbergii and Spacelotheca reiliana and found that the variability in fatty acid composition largely depended on the carbon and nitrogen substrates used.

3.1.2.1 CARBON SOURCE

Carbohydrates are the best carbon substrates for fungal growth. The potential of any organism to be an economic producer of fat is related to its ability to efficiently utilize the available carbon substrate. Species vary in the optimum concentration of carbohydrate as well as the best carbohydrate for lipid production. The three best carbon sources for most fungi are in decending order, glucose > sucrose > fructose. Barnett and Lilly (1950) found that glucose provided abundant growth and sporulation for C. curcubitarum but sucrose and lactose were relatively poor carbon sources. Maltose gave the best yield of mycelium and a high degree of fat accumulation in Mortierella vinacea, whereas glucose was not as good for mycelium production but was as good for fat synthesis (Chesters and Peberdy, 1965). In P. blakesleeanus, Garton et al. (1951) found that lipid production on maltose, fructose and xylose was indistinguishable from that obtained on glucose. Fructose in the medium produced oil with a high

percentage of unsaturated fatty acids while maltose produced an oil rich in saturated fatty acids in S. reiliana (Farag et al., 1983). Lactose was reported to be a poor substrate for lipid production in most organisms but rapid fermentation rates and lactose utilization have been achieved in Candida curvata (Moon and Hammond, 1978).

Increasing the the concentration of glucose or other carbohydrate substrates in the medium can have a marked influence on the fat content of an oleagenous organism as well as on its fatty acid composition. Lipid concentrations increase linearly with increasing glucose concentrations. Chesters and Peberdy (1965) found that a high concentration of glucose resulted in large yields of mycelium with a high fat content in M. vinacea. The ratio of saturated to unsaturated fatty acids produced in Blakeslea trispora varied inversely to the concentration of sugar.

Other compounds, particularly n-alkanes, have been evaluated for their use as sole carbon substrates for fungal growth. Only a few oleagenous yeasts and fungi are able to use such substrates. In essense, the fatty acids of a micro-organism grown on n-alkanes correspond in chain lengths to those of the substrate used. Odd chain fatty acids and shorter chain fatty acids are produced when odd chain or short chain alkanes respectively are used. This concept would not be

suitable for edible microbial oils unless the organism was grown under carbon limited conditions so as to exhaust all traces of the substrate.

3.1.2.2 NITROGEN SOURCE

The best nitrogen source varies from fungus to fungus. In some species organic nitrogen is best for mycelial growth, whereas NH4+ is more efficient for lipid production. Some yeasts accumulate much higher amounts of lipid when an organic nitrogen source is used instead of NH4+ ions (Ratledge, 1984). Garton et al. (1951) reported that fat synthesis in P. blakesleeanus was reduced when ammonium acetate was used as a nitrogen source and growth ceased on ammonium nitrate. In C. curcubitarum, Barnett and Lilly (1950) reported that asparagine and casein hydrolysate were excellent sources of nitrogen while nitrate supported only sparse growth. Chesters and Peberdy (1965) found in M. vinacea that although organic nitrogen substrates produced high yields of mycelium they were inferior to ammonium phosphate for fat accumulation. Molasses hydrolysate and urea were found to be the best for fungal growth in T. ebrenbergii and S. reiliana (Farag et al., 1983). They also found that if the nitrogen source was changed from urea to peptone, the concentration of total unsaturated fatty acids decreased by a factor of 1.6 in

- T. ebrenberii. This was not the case with S. reiliana where total unsaturation for lipids produced from urea and peptone was equal but the fatty acid profiles were quite different.

3.1.2.3 CARBON:NITROGEN RATIO

The carbon to nitrogen ratio is considered to be one of the most important parameters for lipid production. Hesse (1949) reported that the C:N ratio within limits determines the fat content of the cell independently of the concentration of the nutrients. The optimum ratio varies widely from fungus to fungus. Generally, a high C:N ratio favours fat production and a low C:N ratio favours protein production.

Garton et al. (1951) performed two interesting experiments to observe the effect of changing the C:N ratio of the medium. In the first experiment they kept the nitrogen concentration constant and varied the C:N ratio by increasing the glucose concentration. It was found that the lipid production increased slowly at first and at a C:N ratio of 15:1 it accelerated until a maximum value at 25:1 was obtained. In the second experiment, the glucose concentration was kept constant and the nitrogen concentration varied to provide the same C:N ratios as in the first experiment. The results were markedly different. The dry weight, lipid and β-carotene production were basically the same for all C:N

ratios. Garton et al. (1951) concluded that as long as there is sufficient nitrogen present to allow maximum growth the, C:N ratio is not important. Chesters and Peberdy (1965) similarly concluded, after studies on varying the C:N ratio in M. vinacea, that the concentration of the carbon and nitrogen sources is as important as their relative proportions in the control of fat production in fungi.

3.1.3 MINERAL SALTS

The importance of the amount and the composition of mineral salts in fungal growth media is not well understood. Defined media usually include calcium, magnesium, phosphate, potasium, nitrogen and carbon as the major elements with the minor elements as iron, sodium, manganese and zinc. The calcium, sodium and iron may not be necessary although one study showed that as the sodium cloride was increased from 0 - 10 % there was an increase in lipid production.

3.1.4 TEMPERATURE

Reports on the effect of temperature on lipid production seem inconclusive . Fungi are generally classified into three groups according to the their optimum growth temperature requirements. Mesophilic fungi grow best between 25°C and 33°C and psychrophilic fungi between 10°C and 20°C. Thermophilic fungi are defined on the

basis of maximum and minimum growth temperatures (ie. maximum temperature for growth at or above 50°C and minimum at or below 20°C). Thermotolerant fungi can grow near 50°C but below 20°C. Generally, there is an increase in lipid production as the growth temperature increases. This is probably true within certain temperature ranges and this range, as well as the response to variations in temperature, depends on the species. However, lowering the growth temperature of Saccharomyces cerevisiae was accompanied by an increase in total lipid (Hunter and Rose, 1972).

There is a general tendency for fatty acids to become less saturated at lower growth temperatures, although there are often compensating effects in that linoleic acid and linolenic acid increase while oleic may decrease. Shaw (1966a) reported that increasing the culture temperature resulted in a fall in the proportion of GLA, stearic acid and palmitoleic acid in the mycelial lipid. Due to the simultaneous increase in palmitic and and linoleic acids there was little change in the overall degree of saturation in R. arrhizus. He found that that the GLA concentration was unaffected by a rise in temperature. Shaw concluded that there is no evidence to show that the biosynthesis of GLA contributes to the degree of saturation in Phycomycete fungi.

Sumner and Morgan (1969) reported that lowering the growth temperature from 43°C to 25°C increased the synthesis of unsaturated fatty acids in the spores and mycelia of the thermotolerant and thermophilic fungi in the order Mucorales. The lipid content of the mesophiles was found to be lower but contained a greater proportion of the polyunsaturated fatty acids, with a lower proportion of oleic acid.

Since the increase in unsaturation of a lipid lowers its melting point, it has been postulated that the increase in unsaturation is an adaptation to cold environments and is necessary to maintain lipid fluidity (Rattray, 1975; Moon and Hammond, 1978). There are, however, several instances of lipid becoming more unsaturated at higher growth temperatures in fungi and there are others where there is very little effect on fatty acid composition. The confusion may be due to the use of poor batch culture techniques as the oxygen tension in the medium will vary with temperature. At high growth temperatures the amount of oxygen available in the medium is likely to be so low that it causes a decrease in the unsaturation of the fatty acids. It has been suggested that the effect of temperature is primarily on the rates of synthesis and degradation of unsaturated fatty acids. There may be inhibition or repression of different enzymes involved in fatty acid biosynthesis at different temperatures. If the alteration in the degree of

unsaturation were strictly an adaptive response one might expect to find a linear relationship between growth temperature and the degree of unsaturation. However, the data available are insufficient and incomplete and do not clearly demonstrate a linear relationship.

3.1.5 pH

During fungal growth the pH of the medium changes and the degree of change is dependent on the composition of the medium. It is, therefore, difficult to draw conclusions about the effect of pH on fungal growth and fat production. Garton et al. (1951) found that the pH dropped from its original value of 5.00 to 3.70 during the growth of P. blakesleeanus. In some instances after a preliminary drop, a rise was observed and the response varied with the nitrogen source and the C:N ratio of the medium. There are several reports that there is little correlation between lipid production and the pH of the growth medium. Optimum pH values for the growth of most fungi are between 6.0 and 7.0 but this varies from species to species. In general, lipid accumulation, utilization of glucose and in some cases growth is retarded in unbuffered media.

3.1.6 AERATION

There are differing opinions on the importance of culture aeration for lipid production. Mechanical agitation used for culture aeration influences the temperature (3.1.4) and the oxygen tension in the medium. Aerated cultures grow more rapidly and utilize most of the available carbon substrate by the end of the growth period. However, there are no significant differences in the amount and nature of fat in aerated and non aerated cultures. Shaw (1966b), stated that stationary cultures were better for fat production in fungi, although aeration was important for growth.

Oxygen is required for the desaturating enzymes and thus for the dehydrogenation of fatty acids. Therefore, factors affecting its availability must affect the rate of desaturation. In some yeasts a depleted air supply leads to an accumulation of saturated fatty acids.

The method of culture is important as the degree of aeration will vary. Most shake flask experiments will probably be limited to the rate of oxygen transfer into the medium.

3.1.7 VITAMINS

Vitamin deficiencies usually cause a reduction in lipid production, although the relationship is not yet fully established.

A deficiency in pyridoxine, pantothenic acid and biotin results in a reduction in lipid production. Biotin is required as a co-factor in the synthesis of co-carboxylase, the enzyme involved in the chain lengthening process in fatty acid biosynthesis. There is evidence that pyridoxine influences the degree of unsaturation. Nicotinic acid and inosistol deficiency increases lipid production. Other vitamins that may influence lipid production are thiamin, certain amino acids and purines.

Barnett and Lilly (1950) reported that C. curcubitarum is deficient for thiamine and auxotrophic for biotin, inositol and pyridoxine. Growth was very sparse without thiamine added to the medium.

The aim of this chapter was to determine how these environmental factors affect lipid production and particularly the GLA content in the mycelium of C. curcubitarum. By gaining a better understanding of basic physiological aspests, it was hoped that a system for optimal lipid and GLA production by C. curcubitarum could be developed and ultimately utilized for screening mutants in lipid production.

The effect of vitamins on the fungus was not examined as the ultimate aim is to achieve greater yields of GLA in an industrial fermentation and vitamins are too costly to add to the medium.

3.2 MATERIALS AND METHODS

3.2.1 FUNGAL STRAIN

The C. curcubitarum 12997 was obtained from ATCC.

The culture was maintained on GYE-agar plates which were kept at 4°C and transferred once a month. The reason that the cultures were maintained on plates, rather than the usual slants, was that it was found that few or no spores were present on slants. It was therefore difficult to maintain viable cultures on slants. This has also been reported by Barnett and Lilly (1950).

A suspension of spores was kept in distilled water at 4°C and a fresh spore stock was made every three months. A spore suspension was also frozen and used if required as described by Barnett and Lilly (1950).

3.2.2 MEDIA

Media used are listed in the appendix. Shaw's medium (1965) was used in most experiments.

3.2.3 SPORE SUSPENSION PREPARATION

Spore suspensions were prepared from four day old sporulating mycelia grown on GYE/actidione agar media plates. The spores were loosened from the plate with 10ml of sterile distilled water using a glass rod. One

drop of tween 80 per 100ml was added to the distilled water to keep the spores in suspension. The spore suspension was then transferred into sterile standard containers.

GYE/actidione plates were innoculated with 0.1ml of the spore suspension and incubated at RT and 37°C for two days. The plates were examined for bacterial contamination. The spore suspension was checked microscopically for fungal spore contamination.

3.2.4 INNOCULATION AND INCUBATION

Freshly prepared spore stocks were left at room temperature for two days while checking for contamination and a haemocytometer was used to determine the number of spores/ml.

Liquid medium cultures were innoculated with a total of 1×10^6 spores and the cultures were grown in Ehrlenmeyer flasks in a Gallenkamp orbital shaker set at 80 r.p.m. Incubation was at RT.

Agar medium plates (20ml/plate) were innoculated by placing a small piece of mycelium from a four day old culture, grown on GYE/Novobiacin plates, in the centre of the plate. The plates were exposed to alternating light and darkness.

3.2.5 PHYSICAL MEASUREMENTS

3.2.5.1 pH

The pH of agar media was determined using Universal indicator strips (Merck) and that of liquid media using an Orion Research Model 701A/digital Ionanalyser.

3.2.5.2 Glucose Determination

Glucose was determined by glucose oxidation of culture filtrates in a Beckman Glucose Analyser 2.

3.2.5.3 Nitrogen Determination

Nitrogen was determined by the Kjeldahl method (Florence and Milner, 1979).

3.2.5.4 Carotene Determination

ß-Carotene was determined by measureing the absorbance in hexane at 482nm in a Bausch and Lomb Spectronic spectrophotometer (Booth, 1969).

3.2.5.5 Extraction and Analysis of Lipid

3.2.5.4.1 Agar Medium

Three plates of Shaw's agar medium were innoculated as described in 3.2.4 and incubated for four days.

analysis.

Samples of 1 - 2g were used for extraction by the conventional method described in 2.2.2 and samples of 70- 100mg were used for extraction by the HP method as described in 2.2.3.

Two samples of at least 0.5g were used to perform duplicate dry weight analyses as described in 2.2.4.

3.2.5.4.2 Liquid Medium

Shake flasks were innoculated and incubated as described in 3.2.4. Samples for conventional, HP, and dry weight analyses were immediately weighed as described in 3.2.5.4.1.

Samples of 10 - 15g were used for analysis by the conventional method described in 2.2.2.

3.2.6 UNITS

The units used for GLA and fatty acid yields have been described in 2.2.6.

In section 3.3.6, the O2 ratio is defined as the volume of the flask used divided by the volume of medium in the flask.

The efficiency of conversion of glucose to lipid canbe expressed in terms of the fat co-efficient, which is defined as the amount of fatty acid produced per 100g

of carbon source utilized (Chesters and Peberdy, 1965).

All experiments were repeated at least three times and the results shown are representative.

3.3 RESULTS AND DISCUSSION

3.3.1 MEDIA OPTIMIZATION

Various media were innoculated with C. curcubitarum 12997 and incubated at RT for four days. The lipid content of the mycelia was extracted and analysed by the conventional method described in 2.2.2 and the results compared (Table 3.1A and B).

Optimum growth and GLA and lipid production was obtained in Shaw's complete medium (Shaw, 1965). The % of GLA in the fatty acids was 4.3 % higher than that found by Shaw. This could have been due to the substitution of 20g of maltose by glucose or due to the different extraction methods used.

MM1 (Hesseltine, 1954) is a minimal medium and produced the highest GLA and fatty acid yields per 100g dry matter. Growth, however, was inhibited and the medium was therefore not suitable for lipid production.

MM2 (Sumner et al., 1969), also a minimal medium, yielded better growth than MM1, but GLA production was still low and the medium was also excluded as a possible medium for futher studies on lipid production in C. curcubitarum.

Both these minimal media could be used to screen for auxotrophic mutants.

Table 3.1A : The production of lipid and GLA by C. curcubitarum in liquid medium.

MEDIUM	DRY MATTER g/l	% GLA IN F.A.	GLA mg/100g dry mat.	GLA mg/l medium	FATTY ACID mg/100g dry matter	F.A. g/l medium
SHAW`S	4.88	18.3	2138.6	104.3	11.7	0.57
MM1	1.10	18.6	3313.2	36.5	17.8	0.20
MM2	2.20	2.1	141.8	3.1	6.9	0.15

Table 3.1B : The production of lipid and GLA by C. curcubitarum in agar medium.

MEDIUM	DRY MATTER g/l	% GLA IN F.A.	GLA mg/100g dry mat.	GLA mg/l medium	FATTY ACID mg/100g dry matter	F.A. g/l medium
SHAW`S	15.30	18.0	3054.3	467.3	17.0	2.60
GYE	3.7	22.0	1363.6	50.5	6.2	0.23
MM1	5.1	17.9	2682.6	136.8	15.0	0.77

GLA and lipid yields on GYE were low and did not compare with Shaw's medium.

GLA production and the % GLA in the fatty acids was much higher on all the agar media than in the liquid media. This could be due to the increase in oxygen available for growth and lipid production in solid culture.

Shaw's agar medium was the medium of choice for the examination of the environmental factors affecting lipid and GLA production. The medium not only provided the highest lipid and GLA yields, but solid medium is also a convenient medium for genetic studies.

Agar medium should seriously be considered for GLA production on a large scale as there is at least a 4.5 fold increase in the amount of GLA produced per liter of medium. There are problems associated with large scale solid medium cultures, but success has been achieved with the production of citric acid from Aspergillus.
A disadvatages of soild medium is that the culture is easiliy contaminated as trays are left in the open for maximum aeration and C. curcubitarum, in particular, is easily overgrown. In addition, a large amouut of space is required to shelve the trays of agar medium culture.

3.3.2 GROWTH

Liquid and agar medium cultures were innoculated and incubated as described in 3.2.4. Cultures were sacrificed each day for lipid extraction and analysis by the conventional method described in 2.2.2. Three agar media plates were sacrificed in the case of solid medium cultures.

In the first experiment, C. curcubitarum was incubated in liquid medium for 25 days. In the second experiment, agar medium was used and the results on agar and liquid media compared.

The results are shown in Figures 3.2.A and B.

In liquid medium cultures, growth accelerated until day four, reached a maximum at day 11 and then remained constant throughout the 25 day period of incubation. The lipid and GLA production accelerated rapidly until the fourth day and thereafter continued to increase at a steady rate.

Total lipid production reached a maximum at day 14 and thereafter, the total lipid present in the mycelium decreased. The amount of GLA produced, however, reached a maximum at day 19 and was then utilized by the fungus.

β-carotene production peaked at day 19 (five days after lipid production peaked) and then the pigment started to disappear from the mycelium.

Fig. 3.2: Growth and lipid production by C. curcubitarum in liquid (3.2A) and agar (3.2B) media.

Dry weight (g/l),-● ●-; total fatty acids (g/l),-□ □-; GLA production (mg/l),-▲ ▲-; β-carotene production (mg/l),-◇ ◇-; glucose (%) left in the medium,-○ ○-.

Fig. 3.2A:

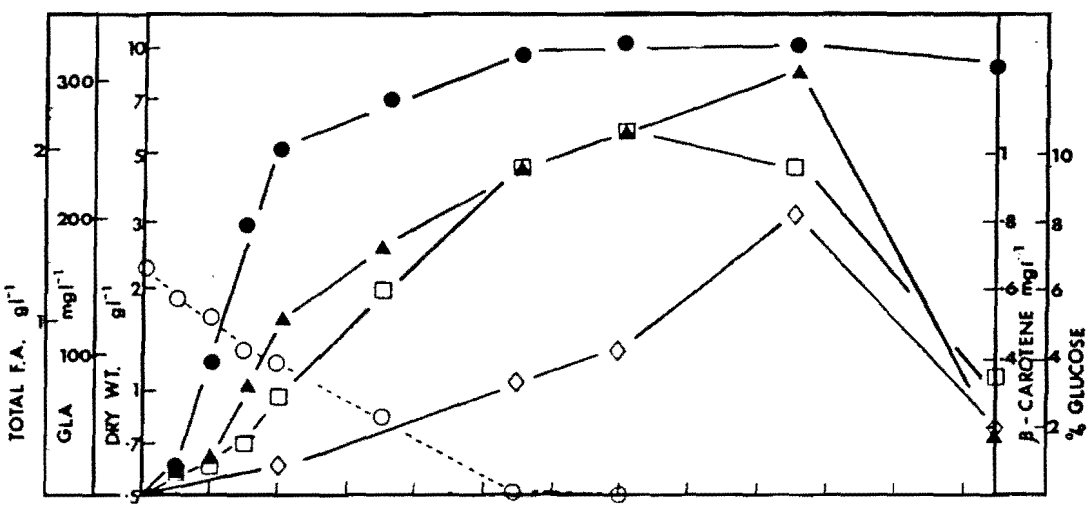


Fig. 3.2B:

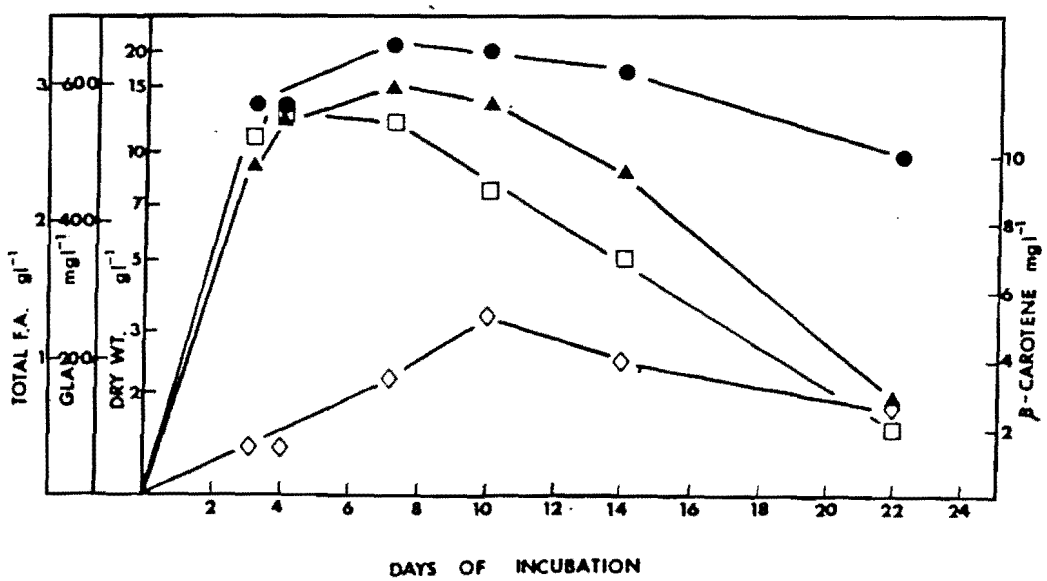
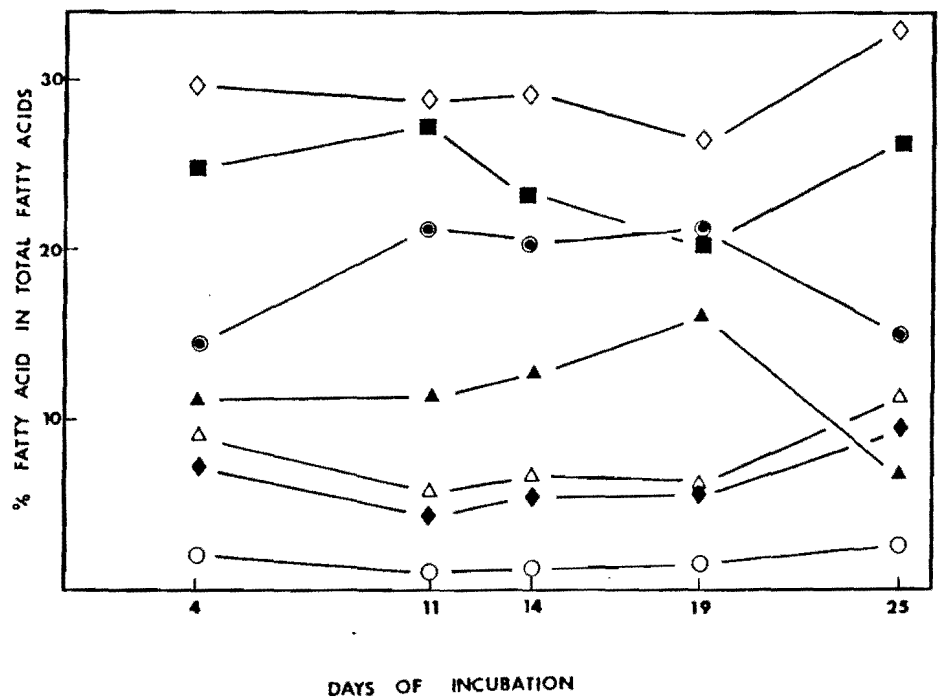


Figure 3.2C : The production of fatty acids by C. curcubitarum.

The % (w/w) of each fatty acid was determined at different time intervals in Shaw's agar medium. Oleic acid, -◇◇-; palmitic acid, -■■-; linoleic acid, -⊙⊙-; GLA, -▲▲-; stearic acid, -△△-; palmitoleic acid, -◆◆-; and myristic acid, -○○-.



The nitrogen concentration in the medium was negligable at day 4 (A. van der Westhuisen, private communication).

Glucose utilization followed a linear pattern until day 11. At day 14 no more glucose could be detected in the medium. It was at this point that the fungus started to utilize the lipid produced. The linear pattern of glucose utilization was also found in M. vinaceae by Chesters and Peberdy (1965). The average uptake of glucose was 0.67g / 100ml medium per day which was much higher than the 0.15g / 100ml medium per day found in M. vinacea.

The pH of the medium initially decreased from 5.5 to 3.5, but started to rise at day 7, to reach a value of 6.19 at day 25.

The growth, lipid and β-carotene production followed the same pattern in agar medium. The lipid production, however, peaked at day 4 and the β-carotene production at day 10. It seems that on agar medium the growth cycle and lipid metabolism was accelerated. This could be due to the increased oxygen available to the mycelium.

White (1962) found that C. curcubitarum accumulated lipid throughout a twelve day period of incubation but did not extend the period of incubation beyond twelve days. When the period of incubation was extended to 25 days, it was found that C. curcubitarum followed a pattern of fat accumulation and depletion similar to P.

blakesleeanus and R. arrhizus which also belong to the order Mucorales (Shaw, 1966a).

As the carbon source became exhausted (day 14 in liquid medium and day 10 in agar medium), the fungus started to utilize the lipid it had accumulated. This supports the theory that the fatty acids are produced primarily as lipid energy reserves. It has been suggested that GLA may have a more specific role as a growth promoting factor (Bernhard et al., 1957, 1958). The fact that the concentration of GLA continues to rise after growth has stopped, could be an arguement against this theory.

The carotene production followed the same pattern as in P. blakesleeanus , with β-carotene production reaching a maximum a few days after lipid production reached its maximum. The pigment disappeared from the mycelium as the culture aged (Garton et al., 1951). The lack of correlation between the time of maximum lipid and carotene synthesis suggests that carotogenesis and lipid synthesis are not linked.

Garton et al found that β-carotene levels reached 0.14 to 0.7 % of the dry weight, whereas in C. cucubitarum a maximum of 0.009 % and 0.03 % was reached in liquid and agar medium respectively. This fungus would therefore not be as suitable for β-carotene production. There is an interest in the production of β-carotene by fermentation for food additives or colour modification

in milk products (Ninet and Renaut, 1979).

As the lipid accumulated the relative proportions of the fatty acids in the lipid varied little (Figure 3.2C). There was an increase in the % GLA in the fatty acids from day 10 to day 19 which suggests that the GLA was conserved while the other fatty acids were utilized as energy reserves. This fall in % GLA was accompanied by a decrease in the % of palmitic acid. White et al. (1952) reported that stearic acid reached a maximum at 56 h and then diminished. The results for stearic acid up to day 11 showed the same pattern, but thereafter, the % stearic acid in the fatty acids increased and was substantially higher by day 25. After day 19, the proportions of the fatty acids in the lipid started to change considerably. There was a marked decrease in the linoleic and GLA content which was accompanied by an increase in oleic, palmitic, stearic and palmitoleic acids. It appears that GLA was initially conserved but then the GLA was utilized while oleic, palmitic, stearic and palmitoleic acids were conseved by the fungus. The significance of these observations is uncertain. It does, however, appear that at a certain stage of fat utilization by C. curcubitarum, the fungus utilizes GLA and linoleic acid before other fatty acids. It seems that in C. curcubitarum GLA is not indispensible and other fatty acids are more important to conserve.

The lipid and GLA levels obtained in the agar medium

cultures are much higher than those reached in liquid medium. This, once again, suggests that large scale production on solid media should be seriously considered.

In liquid medium, the amount of GLA produced at day 11 was four times the amount produced at day 4. It may be advantageous to prolong the fermentation time in large scale production, although this does increase the risk of contamination.

3.3.3. CARBON AND NITROGEN SOURCE

The effect of using different carbon and nitrogen sources was studied by researchers at National Chemical Products (A. van der Westhuisen, private communication).

They found that, of the carbohydrate substances tested, glucose produced the best growth and the highest GLA and lipid yields.

The best nitrogen source was peptone and specifically Lab M Proteose peptone, which provided better yields than other brand names. It was for this reason that Lab M Proteose peptone was used in Shaw's medium in all experiments.

3.3.3.1 CARBON:NITROGEN RATIO

Two experiments, similar to those performed by Garton *et al*. (1951), were performed to examine the effect of the C:N ratio of the medium on GLA and lipid production (3.1.2.3).

In the first experiment, the C:N ratio was varied by keeping the nitrogen concentration constant and varying the glucose concentration.

In the second experiment, the glucose concentration was kept constant and the nitrogen concentration varied to provide the same C:N ratios as in the first experiment. Three agar medium plates for each C:N ratio were

innoculated and incubated as described in 3.2.4 and the lipid extracted and analysed by the HP method described in 2.2.3.

The values obtained for dry weight, lipid and GLA were plotted against the C:N ratio of the medium (Figs. 3.3A and B).

The results were very similar to those obtained by Garton *et al*. (1951).

The effect of increasing the glucose concentration was marked at low C:N ratios and the lipid and GLA production increased rapidly. After a C:N ratio of 15:1, increasing the amount of glucose in the medium had little effect on lipid production (Fig. 3.3A).

In the second experiment, reducing the C:N ratio of the medium by increasing the nitrogen concentration had a less significant effect on the dry weight or lipid production by *C. curcubitarum* (Fig. 3.3B). The values obtained for dry weight decrease at high C:N ratios. At these ratios the nitrogen concentration was insufficient to produce maximal growth. The concentration of GLA and lipid in the mycelium (i.e. per 100g dry matter) increased slightly and the amount produced remained more of less constant. This was not observed by Garton *et al*. who reported that the lipid produced decreased with the growth in *P. blakesleaanus* at high C:N ratios. If the C:N ratio in the medium had been increased beyond 50:1,

Fig. 3.3: The effect of the C:N ratio on the growth, GLA and lipid production in C. curcubitarum.

In the first experiment the nitrogen concentration was kept constant and the glucose concentration was varied (Fig. 3.3A). In the second experiment the glucose concentration was kept constant while the nitrogen concentration was varied to give the same C:N ratios as in the first experiment (Fig. 3.3B).

Dry weight (g/l), -● ● -, total fatty acids (g/l), -□ □ -; GLA produced (mg/l), -▲ ▲ -, pH of the medium after incubation, -▽ ▽ -.

Fig. 3.3A:

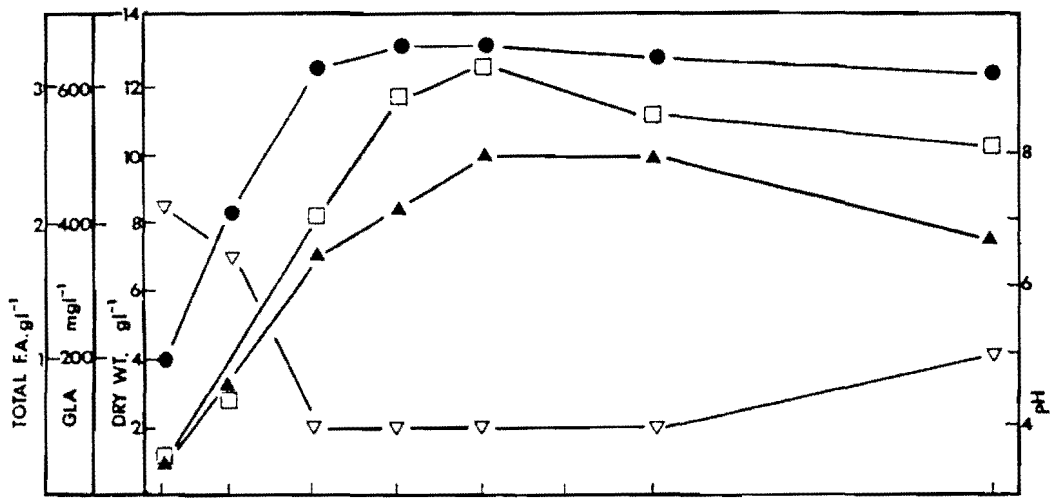
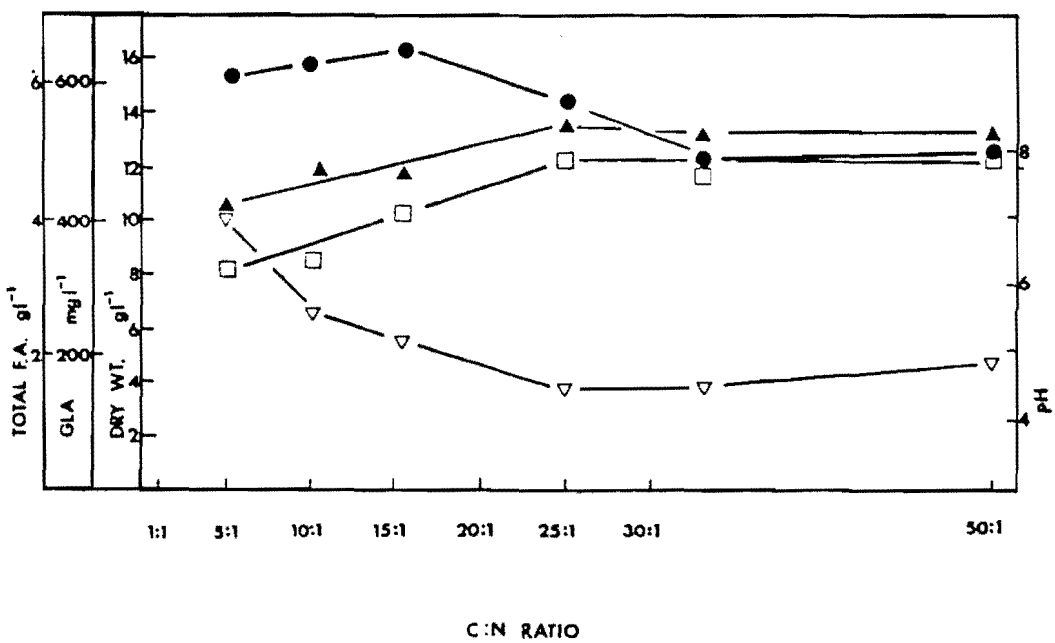


Fig. 3.3B:



the same fall might be found in C. curcubitarum.

From the pH changes in the medium it seems that the excess nitrogen is deaminated and excreted into the medium.

The maximum GLA production occurred at a C:N ratio of between 20:1 and 30:1. Shaw's medium has a C:N ratio of 22:1 and the medium is, therefore, well within this range. This confirmed that the medium is suitable for the study of lipid production in C. curcubitarum.

Similar results were obtained when the experiments were repeated in liquid medium (data not shown).

The results obtained in this section support the conclusion drawn by Garton et al. (1951) that the concentration of the carbon and nitrogen source is as important as their relative proportions. This view is in agreement with Chesters and Peberdy (1965), but in contrast to that of Hesse (1949; section 3.1.2.3) and Weete (1974), who states that " the C:N ratio of the medium is the most important nutritional parameter for lipid production by fungi ".

3.3.4 EFFECT OF TEMPERATURE

Shaw's agar plates were innoculated and incubated at 4 °C, RT, 30 °C, 35 °C and 48 °C.

Six plates were harvested for extraction and analysis by the conventional method described in 2.2.2. The results are tabulated in Table 3.4A and B.

The fungus did not grow at 4°C or 48°C and optimum growth was achieved at 30°C. Barnett and Lilly (1950) reported that mycelial growth decreased below 25°C. They made no attempt to determine the maximum temperature which permitted growth. The facilities available for this research did not permit a stepwise increase in incubation temperature, but the maximum temperature for growth must lie between 36 °C and 48 °C. C. curcubitarum would be classified as a mesophilic fungus.

Microscopic examination of the cultures showed that there were marked differences between the cultures grown at RT, 30 °C and 35 °C.

Cultures grown at RT had the most spores, the least orange-yellow pigment and contained mature sporangiophores with sporiangioliferous heads. Cultures grown at 30 °C contained less spores, were more orange-yellow in colour and no sporiangioliferous heads were present. Swellings in the mycelium appeared to be filled with the pigment. Cultures grown at 35 °C had no spores

which agrees with Barnett and Lilly's (1950) report that the maximum temperature for sporangium formation in C. curcubitarum was 34°C. A large number of swellings in the mycelium were filled with pigmemt. The whole colony appeared a much darker orange-yellow colour and when stained with Sudan black (4.3.1), the mycelium incubated at 35°C seemed to contain the most fat. It was initially thought that if this increase in pigment was indicative of increased lipid production, the colour could be used to screen for mutants in fat production. Analysis, however, showed that the highest yield of lipid/100g dry matter was at 30°C. The highest carotene yield/100g dry matter was at 35°C which explains the darker appearance of the colony. The stain was most likely staining the carotene as well as the lipid in the mycelium.

The optimum temperature for GLA production was 30°C. This was mainly due to the increase in growth at this temperature and there was also a slight increase in the % GLA in fatty acids at 30°C. The highest yield/100g dry matter for both lipid and GLA production occurred at RT but growth was inhibited as it was at 35°C.

It appears that there was an increase in the overall unsaturation of the fatty acids at lower temperatures. This supports the theory that the decrease in unsaturation at lower temperatures may be a means of adaptation to cold environments (3.1.4.),

Table 3.4A : The effect of temperature on GLA and lipid production by C. curcubitarum.

TEMP.	DRY MATTER g/l	% GLA IN F.A.	GLA mg/100g dry mat.	GLA mg/l medium	FATTY ACID g/100g dry matter	F.A. g/l medium
RT	15.2	13.6	3484.7	530.4	25.61	3.9
30 °C	19.9	17.5	2833.0	563.5	16.20	3.2
35 °C	13.1	15.7	2840.7	372.1	18.08	2.4

Table 3.4B : The effect of temperature on carotene production by C. curcubitarum.

TEMP.	CAROTENE mg/100g	CAROTENE µg/ml
RT	9.8	3.0
30 °C	13.6	5.4
35 °C	16.0	4.2

Table 3.4C: The effect of temperature on the ratio of saturated to unsaturated fatty acids produced by C. curcubitarum.

FATTY ACID	RT	30°C	35°C
MYRISTIC (14:0)	1.2	1.4	1.7
PALMITIC (16:0)	26.7	27.7	29.8
PALMITOLEIC (16:1)	3.2	2.7	2.6
STEARIC (18:0)	5.8	4.9	5.8
OLEIC (18:1)	30.6	29.1	23.6
LINOLEIC (18:2)	19.0	19.8	20.6
GLA (18:3)	13.6	17.5	15.7
%SATURATION	33.7	33.9	37.3
%UNSATURATION	66.4	66.1	62.8

although the decrease in unsaturation from RT to 35°C was only 3.6 %.

The changes in the proportions of indivdual fatty acids were different to those reported by Shaw (1966a, 3.1.4). It was found, for example, that the proportion of GLA remained unaffected by an increase in temperature in R. arrhizus, whereas in C. curcubitarum the proportion of GLA actually increased (Table 3.4C). Shaw stated that there was no evidence that the biosynthesis of GLA contributes to the overall saturation of the lipid in Phycomycete fungi the results in this section support this statement.

3.3.5 EFFECT OF pH ON GLA PRODUCTION

The pH of Shaw's agar medium plates was adjusted after autoclaving using Universal indicator strips (Merk). The plates were innoculated as described in 3.2.4 and after incubating at RT for four days the mycelia were harvested. Extraction and analysis of the lipid was performed by the HP method described in 2.2.3. The results are shown in Table 3.5.

Growth on the solid medium plates was totally inhibited at pH 3. The growth and GLA production was retarded at pH 4 and pH 8, but the lipid yields/100g dry matter were less affected by these pH values.

The best growth occurred from pH 5 to pH 7. The highest yield/l medium and per 100g dry matter for GLA was obtained at pH 5 and pH 6 but the highest total fatty acid yield was at pH 7.

The experiment was repeated in liquid medium and similar results were found (data not shown). The only difference being that the growth was not inhibited at pH 3, although GLA and lipid levels in the mycelia were very low.

The pH of the media decreased by a point during incubation, except at an initial pH value of 4.0 where the pH decreased to 3.5.

Table 3.5 : The effect of pH on lipid and GLA production by C. curcubitarum

PH	DRY MATTER g/l	% GLA IN F.A.	GLA mg/100g dry mat.	GLA mg/l medium	FATTY ACID g/100g dry matter	F.A. g/l medium
4.0	4.5	9.0	2426.7	6.6	27.1	1.22
5.0	12.5	11.4	3327.0	25.0	29.2	3.65
6.0	12.7	11.4	3316.1	25.1	29.0	3.68
7.0	12.2	9.2	3153.8	22.9	34.7	4.23
8.0	5.5	10.9	2402.9	7.9	22.0	1.21

In all experiments performed in this thesis, the pH of of Shaw's medium was adjusted to 5.5 before autoclaving and the results show that this is within the optimum pH range for growth and lipid production in C. curcubitarum.

Growth and GLA production was inhibited at pH values below 5.0 and since the pH of the medium decreased during incubation, pH control would be essential during large scale fermentation. As the GLA production was high at pH 6, it may be advantageous to adjust the pH of Shaw`s medium to 6.0 or even 6.5 before innoculation.

3.3.6 EFFECT OF AERATION

Different volumes of Shaw's medium were used in 500 ml flasks to provide O2 ratios of 10 to 1.25 (see 3.2.6 for definition). Each flask was innoculated with a total of 1×10^4 spores and incubated as described in 3.2.4. The mycelia was harvested after a four day incubation period and the lipid extracted and analysed by the conventional method described in 2.2.2.

The medium was analysed for glucose after incubation so that the glucose utilization and the efficiency of conversion to fat could be determined. The efficiency of conversion to fat can be described in terms of the fat co-efficient defined in 3.2.6.

Two litre flasks containing 600ml Shaw's media (O2 ratio of 3.3) were also innoculated and stationary and shaking cultures compared (Table 3.6C and D).

Growth, the efficiency of conversion of glucose to fat and the GLA and lipid production increased as the O2 ratio increased (Fig. 3.6A).

The fat co-efficient increased from 3.21 at an O2 ratio of 1.25 to 12.77 at an O2 ratio of 5 (Table 3.6B). The optimum O2 ratio for GLA/100g dry matter and % GLA in the fatty acids was 5. The leveling off of the GLA curve after the O2 ratio of 5 may be due to the Δ-6 desaturase enzyme becoming saturated. Nevertheless, due to the

increased growth, the greatest amount of GLA produced per litre of medium was at the highest O2 ratio (10). The increase in the GLA and lipid production seems to be mainly due to the increased growth found at higher oxygen ratios.

β-Carotene production increased dramatically from 591.0 ug/l at an O2 ratio of 5 to 2194.0 ug/l at an O2 ratio of 10. This increase is, therefore, not only due to the increase in growth and the increase in oxygen seemed to have a direct effect on carotogenesis.

When the stationary and shaking cultures (O2 ratio 3.3) were compared, the fat co-efficient and the production lipid and GLA were very much lower in the stationary culture. This is in contrast to Shaw (1965) who suggested that stationary cultures are often preferable to shaking cultures for maximum fat yield. The increase in lipid production in shaking cultures can be attributed to the 47 fold increase in growth.

The % GLA in the fatty acids was, however, higher in stationary cultures. This was contrary to what was expected as there would be far less oxygen available for the enzymes involved in desaturation. White et al. (1962) reported the presence of an unidentified fatty acid, X4, in C. curcubitarum which eluted after linoleic acid on the chromatograph. This fatty acid was most likely GLA. The % of X4 in the fatty acids was also

Fig. 3.6A : The influence of the O2 ratio on the growth, fatty acid and β-carotene production by C. curcubitarum.

Dry weight (g/l), -● ●-; total fatty acids (g/l), -□ □-; GLA (mg/l), -▲ ▲-; β-carotene (mg/l), -◇ ◇-.

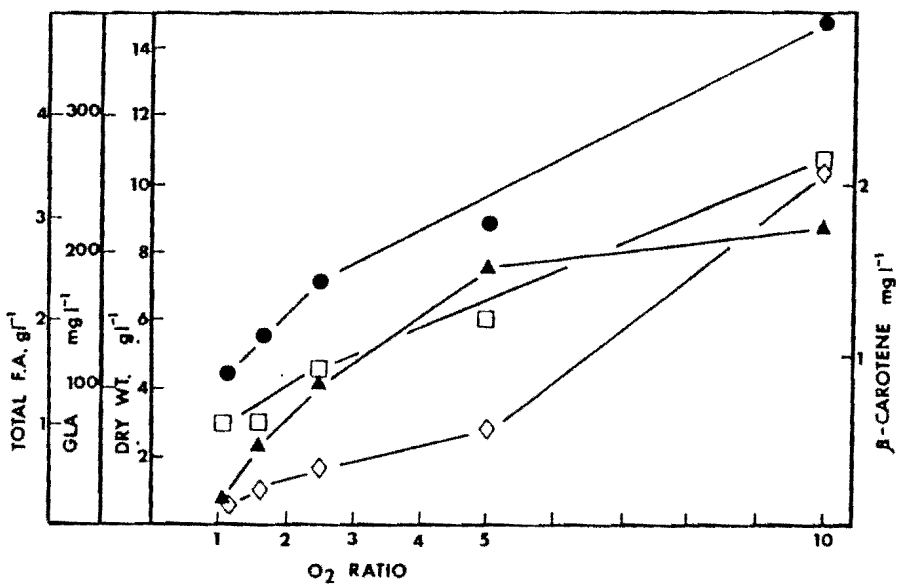


Table 3.6B: The influence of the O2 ratio on the efficiency of conversion of glucose to fat by C. curcubitarum.

O2 RATIO	FAT CO-EFFICIENT
10	7.41
5	12.77
2.5	4.67
1.7	3.17
1.25	3.21

Table 3.6C : Fatty acid and GLA production in stationary and shaking cultures by C. curcubitarum.

O2 RATIO	DRY MATTER g/l	% GLA IN F.A.	GLA mg/100g dry mat.	GLA mg/l medium	FATTY ACID g/100g dry matter	F.A. mg/l medium
3.3 shak.	6.6	7.5	1352.2	89.2	18.0	1193.5
3.3 stat.	0.14	13.8	1393.0	1.9	10.1	13.9

Table 3.6D : Carotene production and glucose utilization in shaking and stationary cultures by C. curcubitarum.

O2 RATIO	CAROTENE mg/100g dry mat.	CAROTENE µg/l medium	FAT CO-EFF.
3.3 shak.	322.5	4.9	2.59
3.3 stat.	23.0	16.9	0.13

Table 3.6E : The influence of the O2 ratio on the ratio of saturated to unsaturated fatty acids produced by C. curcubitarum.

O2 RATIO	10	5	2.5	1.7	1.25	3.3 stat.	3.3 shak.
			fatty acid analysis % w/w				
MYRISTIC (14:0)	1.9	2.7	1.9	1.8	3.3	-	2.7
PALMITIC (16:0)	27.9	23.7	23.8	23.8	23.5	24.0	27.0
PALMITOLEIC (16:1)	4.2	12.4	6.9	6.2	7.1	-	12.4
STEARIC (18:0)	6.9	7.2	7.5	8.5	14.3	17.5	7.1
OLEIC (18:1)	23.8	24.5	24.7	26.5	20.8	23.7	24.5
LINOLEIC (18:2)	24.2	22.1	23.2	19.1	22.7	21.0	22.1
GLA (18:3)	11.2	7.5	12.0	14.2	8.3	13.8	7.5
% UNSAT.	63.4	66.5	66.8	66.0	58.9	58.5	66.5
% SAT.	36.3	33.5	33.2	34.0	41.1	41.5	33.5

found to be higher in static culture (19.3 % in static culture and 9.3 % in shaking culture) which agrees with these findings.

There was a decrease in the % unsaturation at lower O2 ratios and this decrease was in the same proportion as the decrease found when comparing shaking and stationary cultures at an O2 ratio of 3.3 (Table 3.6E). This can be attributed to the decrease in oxygen available to the mycelium.

Increasing the oxygen concentration in the medium had a marked effect on the growth and therefore the GLA production by C. cucubitarum.

The increase in growth and GLA production on agar medium as compared to liquid meidum can also explained by the increase in available oxygen to the mycelium (3.3.1).

3.3.6 ADDING LINOLEIC ACID TO THE MEDIUM

It was decided to add increasing quantities of linoleic acid to the growth medium to determine whether C. curcubitarum would take the oil up and convert the excess linoleic acid into GLA. Linoleic acid was added to Shaw's medium in quantities ranging from 0.1 - 5 %. It was found that the growth and GLA production was inhibited for all concentrations of linoleic acid added to the medium. Perhaps better results would be obtained if the linoleic acid was added as a salt.

3.4 GENERAL CONCLUSION

Shaw's agar medium is suitable for the study of GLA and lipid production in C. curcubitarum and could be used as a medium for screening mutants in fat production. The medium contains glucose, which is the best carbohydrate source for maximum fat yields in the fungus; the C:N ratio is 22:1, which is well within the optimum range established; the pH is 5.5, which is also within the optimum pH range. Agar medium not only allows for maximum aeration to the mycelium, but is convenient for genetic studies.

The significance of the trace elements added to the medium or the effect of various vitamins on GLA and lipid production has not been studied. This could be an area for futher research in order to gain a better understanding of all the environmental factors affecting the GLA and lipid production by C. curcubitarum.

Abundant growth is the predominant requirement for good GLA production. The highest yields/litre medium were always found where growth was at its maximum. (That is, at the optimum C:N ratio, temperature, pH and maximum culture aeration). It did not necessarily follow that the highest GLA per 100g dry matter or % GLA in the fatty acids produced, was maximum under these conditions. In large scale production, therefore, maximum growth should be of prime importance.

The role of GLA remains uncertain, although the lipid produced seems to be an energy reserve as it is utilized by the fungus as the carbon source becomes depleted from the medium.

It was established that carotogenesis and lipid production are not directly linked. The pigment can, therefore, not be used as a suitable method for screening mutants in fat production. Some other screening method must be developed for a genetic programme to be sucessful.

CHAPTER 4

GENETIC STUDIES ON *C. curcubitarum*.

SUMMARY

The induction of mutations and the isolation of *C. curcubitarum* mutants with increased GLA production was investigated. A suitable medium for assaying fungal colonies of *C. curcubitarum* was developed. Exposure of the spores to 0.1 mg/ml NTG in distilled water for 30 - 60 mins resulted in 0.1 - 10 % survival of the spores. Staining the lipid globules in the mycelium (using 3 % sudan black in ethane diol) was investigated as a method for screening for mutants in lipid production but was not suitable. Four mutants in GLA production were isolated by screening carotene mutants which were easily distinguishable from the WT on the asssaying medium. The link between the increase in GLA production and the increased carotogenesis in the mutants was co-incidental and could be due to multiple mutations induced by NTG. All the mutants in GLA and lipid production were unstable and reverted.

4.1 INTRODUCTION

Relatively little work has been done on the genetics of fungi in general and even less in the order Mucorales. There are no reports on the genetics of C. curcubitarum. An example of the Mucorales which has been studied from the genetic aspect is Phycomyces.

The genetics of Phycomyces has been reviewed by Cerda-Olmedo (1974) and Bergman et al. (1969) and the following information, unless otherwise quoted, has been extracted from these reviews.

The study of the genetics of Phycomyces was begun by Burgeff in 1911, who gave up in 1928 due to difficulties in finding an appropriate marker, sluggishness in the sexual cycle and the irregulatities in the results. The research in sensory physiology has recently stimulated new interest in the genetics of Phycomyces.

Morphlogical mutants are frequently found and often occur spontaneously. Convenient markers are provided by inhibitory drugs, or requirement of special nutritional supplements.

Mutations that block the synthesis of β-carotene and result in the loss of the yellow colour are found on three loci. Mutants carA are white and lack forty-carbon compounds. Mutants carB are white and accumulate phytoene. Mutants carR are red and accumulate lycopene.

Other *car* mutants produce more carotene than the wild type and have a brighter yellow colour.

There are mutants which are resistant to crystal violet (*xtv*), nystatin (*nys*), amphotericine B (*amp*), and canavine (*can*). Those isolated after plating large numbers of untreated spores are usually dominant.

Auxotrophs can be isolated after strong mutagenic treatments. A number of stable auxotrophic mutants have been isolated and their nutritional requirements (amino acids, vitamins, adenine) have been determined. Many mutants isolated are, however, unstable and revert to prototrophy. Several counterselection procedures have been tried, but none of these have been particularly successful.

Mutants characterised by altered phototropism are designated *mad* and are isolated because of the inability of their sporangiophores to turn downward when grown in a glass-bottom box, where all the light comes from below.

To obtain most of these mutants, the spores are treated with the mutagen NTG. Heat-shocked spores of *Phycomyces* suspended in a solution of NTG (0.1 to 1 mg/ml) in buffer (acetate or tris(hydroxymethyl)amino-methane-maleic, pH 5 to 7) for 15 - 80 min and washed in distilled water gave a high yield of mutants and a survival rate sufficiently low to produce few

heterokaryons. Under these conditions NTG may induce base changes which tend to be clustered in the replicating DNA at the time of treatment. Since the mutant isolation procedure of Phycomyces required the growth to maturity of all spores to be screened, Bergman (1973) reported that the isolation procedure was not extremely efficient. The isolation of mutants requires treatment with high levels of potent mutagens which unavoidably results in the simultaneous presence of several mutations in each nucleus.

The multinucleate nature of most of the spores demands that the mutagenic treatments be coupled with the death of most of the nuclei so that enough functionally uninucleate spores are produced for the detection of recessive mutants or that the few uninucleate spores may be isolated and tested.

Before any genetic programme can be started there are a few basic requirements that have to be met. The first of these the development of a medium suitable for assaying mycelial fungal colonies which can be spread over the surface of an agar plate. Yeast-like growth has been obtained in Mucor genevensis when the fungus is grown under anaerobic conditions in the presence of 0.1 % phenethyl alcohol (Gordon et al., 1971). Mucor rouxii is reported to develop yeast-like cells at high levels of CO2 and hexose sugars (glucose being the most effective) under anaerobic conditions (Bartnicki-Garcia, 1968).

The second requirement is to find a suitable mutagen and the conditions which produce 0.1 - 10 % survival.

Thirdly, the success of any genetic programme depends on finding a suitable screening technique. An attempt was to stain the lipid globules in the mycelium. Lien (1981) reported a highly efficient cytochemical staining technique, suitable for screening and evaluating a large number of algal species for their oleaginous capacity. Samples of the algae were pulse-stained with CI Basic Blue 12 (Nile Blue) under mild alkaline or neutral conditions and the oil droplets were readily visualized as yellowish-orange structures, while the more polar constituents were stained deep blue. The fact that an increase in the lipid globules may be a good indication of increased GLA production has already been demonstrated (2.7.2).

The next attempt was to screen for carotene mutants as it first appeared that there may be a connection between increased lipid production and carotogenesis. The carotene mutants are easy to distinguish due to marked differences in the colour of the colonies. If a link between lipid production or even GLA production and carotgenesis could be found, this would provide a convenient means of screening for mutants.

The aim of this investigation was to do the basic ground work needed before a genetic program for C. curcubitarum

could be developed.

4.2 MATERIALS AND METHODS

4.2.1 FUNGAL STRAIN

The strains used were C. curcubitarum 12997, 2744, 46105 and 46106 which were obtained from ATTC. The cultures were maintained as described in 3.2.1. When not otherwise stated, C. curcubitarum 12997 was used.

4.2.2 MEDIA

All media used are listed in the appendix.

4.2.3 SPORE SUSPENSION PREPARATION

Spore suspensions were prepared as described in 3.2.3 and freshly prepared stocks were used for mutagenesis.

4.2.4 STAINING TECHNIQUE

The myceium from four day old cultures of the different strains of C. curcubirarum was soaked in various stains at concentrations of 0.3 - 3.0 %, for up to 3 h.

The mycelium was examined microscopically.

4.2.5 MUTAGENESIS

Freshly prepared spore stocks were prepared and incubated with 0.1 mg/ml NTG in distilled water at 22 °C. Samples were withdrawn at various time intervals

and the NTG was removed by centrifuging the samples at 4000 r.p.m. for 3 min . The spores were resuspended in sterile distilled water, plated out in the assay medium and incubated at RT for four days.

4.2.6 METHOD OF EXTRACTON AND ANALYSIS OF LIPID AND CAROTENE

The lipid was extracted and analysed by the conventional and the HP method of extraction and analysis as described in 2.2.2 and 2.2.3, respectively.

β-carotene levels were determined as described in 3.2.5.4.

4.2.7 DRY WEIGHT ANALYSES AND UNITS

As described in 2.2.4 and 2.2.6, respectively.

4.3 RESULTS

4.3.1 DEVELOPING A STAINING TECHNIQUE

The lipid globules in the mycelium of C. curcubitarum were clearly visible in the mycelium using slide cultures and impression mounts even without staining the lipid.

The following stains were used as described in 4.2.4: sudan black, oil red and nile blue in the solvents; acetone, acetone:water (1:1 v/v) and ethylene glycol. The mycelium was examined microscpically at half-hourly intervals and the results compared. Using all the stains, the lipid globules were clearly visible in the mycelium.

The most effective staining was achieved by soaking the mycelium in 3% sudan black in ethylene glycol for an hour. An increase in the blue-black colour in the mycelium correlated well with an increase in lipid production.

The staining technique was tested using the mycelium of C. curcubitarum 12997 , 2744, 46105 and 46106. In addition, C. curcubitarum 12997 grown at RT, 30°C and 35°C and used to test the technique. The amount of lipid stained was recorded by using +; ++ and +++, where an increase in the amount of lipid stained was indicated by an increase in the number of +'s used.

The results obtained with the various strains of C. curcubitarum correlated well with the results obtained when the lipid was extracted from their mycelia, using the conventional method (2.2.2). However, the results of staining C. curcubitarum 12997 grown at RT, 30°C and 35°C indicated that the lipid production increased with an increase in temperature. When the actual lipid levels were determined, it was found that the highest yield of fatty acid (g/100g DM) was actually at RT, then at 35°C and then 30°C (3.3.4). It seems, therefore, that the increased levels of carotene were being stained by the sudan black (the amount of carotene in mg/100g DM increased with an increase in temperature; 3.3.4) and the results obtained using the staining technique were misleading.

4.3.2 ASSAY MEDIUM FOR C.curcubitarum.

Various atempts were made to inhibit or contain the mycelial growth of C. curcubitarum.

The methods used to induce a yeast-like growth in M. genevensis and M. rouxii were not suitable for C. curcubitarum as the fungus would not grow under anaerobic conditions (4.1). The addition of phenethyl alcohol (0.05 %) to Shaw's medium inhibited mycelial growth but it was not sufficient to be used as a assay medium. The addition of higher concentrations of phenethyl alcohol resulted in total inhibition of spore germination. The phenethyl alcohol is also highly corrosive and was abandoned as a possible means to inhibit the mycelial growth of C. curcubitarum.

The most effective medium found for assaying fungal colonies of C. curcubitarum was to adjust the pH of the GYE (2 % agar) medium to 4.0 after autoclaving and add 0.1ml of 10 % tartaric acid per 15 ml medium prior to pouring the plates (see appendix). Single isolated colonies could be counted after incubation at RT for four days. The average % germination in the counting medium was 50 % as compared to 80 % in GYE and the formation of an orange pigment (carotene) was noted. Oxoid No. 1 agar had to be used in the medium otherwise the agar would not set at the low pH.

4.3.3 MUTAGENESIS

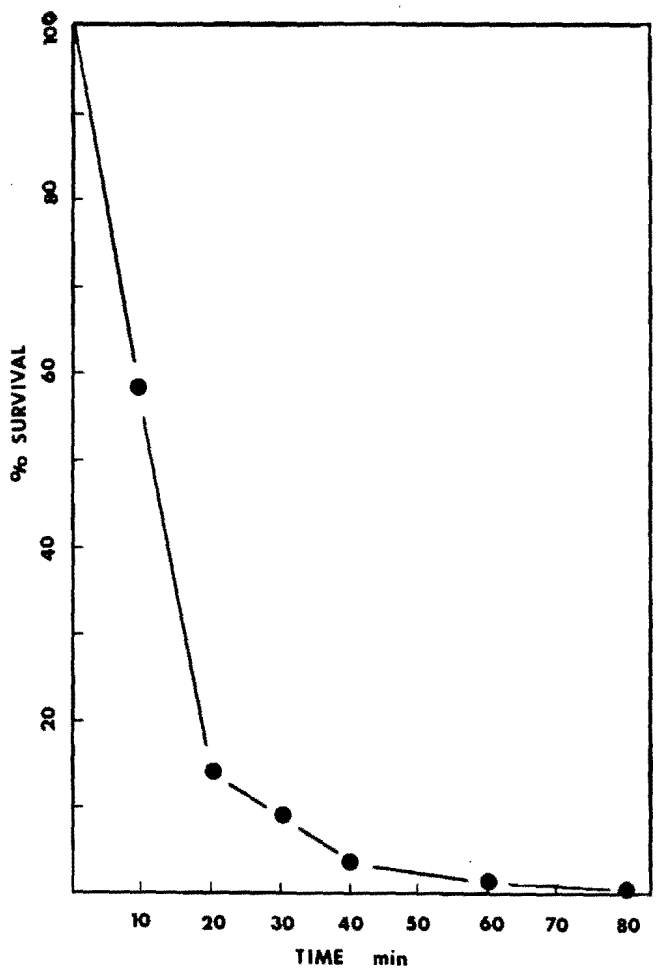
The results of mutation with U.V. light were not reproducible as the spores of C. curcubitarum are black and thus not transparent to the U.V. light.

Spores were mutated as described in 4.2.5 and a reproducible survival curve was obtained (Fig. 4.1). Survival levels of 0.1 - 10 % were obtained at 30 - 60 mins.

Heat activated spores (15 min at 48°C) were not used as germination of the spores was totally inhibited.

Fig. 4.1: Survival of C. curcubitarum after treatment with NTG.

Spores were exposed to NTG for X prior to plotting on Y.



4.3.4 MUTANTS

All mutants were obtained after exposing the spore stock to 0.1 mg/ml N.T.G for 40 min as described in 4.2.5. The mutated spores were allowed to germinate in the assay medium and carotene mutants were selected (after incubation for four days at RT) on the basis of a change in the colour of the colony as compared to the WT.

The selected mutants were grown on either Shaw's agar medium or Shaw's liquid medium, at RT for four days as described in 3.2.4. The lipid was extracted and analysed and the results compared to the WT.

The mutants that showed altered lipid production are shown in Table 4.2A and B. The carC2 series of mutants were obtained by mutating the spores of carR1 and were selected by the method for screening for mutants in lipid production developed in section 2.3.3.1.

The most interesting of the mutants screened was carR1, which was red in colour and showed 150 % increase in GLA production (Table 4.2A and B). The mutant again showed a similar increase in GLA production after growth in Shaw's liquid medium. However, the mutant was unfortunately unstable and first lost its ability to produce the high levels of GLA and subsequently the red colour disappeared. This suggested that the mutation was a double mutation and that the red colour was

co-incidental.

The other mutant that was studied in more detail was carC1, which showed an increase of 57 % in GLA production in liquid medium (Table 4.2A) and a 28 % increase in GLA production on Shaw's agar medium (Table 4.2B). Again, the mutant was an unstable double mutant which first lost its ability to produce increased levels of GLA and then the bright orange colour.

The carotene levels of both car-R1 and car-C1 were increased by 500 % (Table 4.2A). The growth patterns of both these mutants were examined on Shaw's agar medium and compared to the WT. The mutants followed a pattern of fat accumulation and depletion similar to the WT. The carotene production was, however, different as in both mutants the carotene production remained constant throughout the 22 day incubation period. The carotene did not start to disappear from the mycelium at day 10 as it did in the WT (data not shown).

Once the mutants had reverted the lipid and GLA levels were compared to the WT and the results are shown in Table 4.2C.

The mutants carC2-8 and carC2-9 were even less stable than carR1 and carC1 as they had already reverted prior to growth on Shaw's agar medium.

Table 4.2A: Mutants of C. curcubitarum with increased GLA and carotene production when grown in Shaw's liquid medium. The lipid was extracted and analysed by the conventional method (2.2.2). CarR1 and carC1 were grown in 600ml medium in 2L flasks. CarC2-8 and carC2-9 were grown in 200ml medium in 500ml flasks. Cultures were innoculated with 1.6 x 10^6 spores/ml.

MUTANT	COLOUR	DRY MATTER g/l	% GLA in F.A.	YIELD GLA mg/l	YIELD F.A. g/l	YIELD CAROTENE mg/l
carR1	red	5.50	24.6	226.7	0.92	2.48
carC1	bright orange	5.05	21.6	140.3	0.65	2.50
WT	slightly orange	6.60	7.4	89.2	1.20	0.05
carC2-8	orange	5.65	20.8	131.1	0.63	-
carC2-9	orange	6.65	23.7	182.7	0.77	-
WT	slightly orange	6.15	7.3	116.9	1.61	-

Table 4.2B: Mutants of C. curcubitarum with increased GLA and carotene production when grown on Shaw's agar medium. The lipid was extracted and analysed by the HP method (2.2.3).

MUTANT	COLOUR	DRY MATTER g/l	% GLA in F.A.	YIELD GLA mg/l	YIELD F.A. g/l
carR1	red	12.2	20.0	1217.8	6.10
carC1	bright orange	16.1	23.7	617.4	2.60
WT	slightly orange	14.2	18.5	481.5	2.60
carC2-8	orange	9.0	21.9	175.5	0.80
carC2-9	orange	7.2	23.6	198.1	0.84
WT	slightly orange	5.8	19.9	211.2	1.06

Table 4.2C: GLA production by car mutants which have reverted and the WT culture.
The cultures were grown in 600ml Shaw's medium in 2L flasks and the lipid was extracted and analysed by the HP method (2.2.3).

MUTANT	DRY MATTER g/l	% GLA in F.A	YIELD GLA mg/100g dry mat.	GLA mg/l medium	YIELD F.A. g/100g dry matter	F.A. g/l medium
car-C1	5.23	20.0	2641.2	138.1	13.2	0.69
car-R1	4.63	10.3	2485.6	115.1	24.2	1.12
WT	4.83	10.8	2731.5	131.9	25.2	1.22

4.3.5 DISCUSSION

An increase in the lipid globules in the mycelium of C. curcubitarum could be a good indication of increased GLA production (2.7.2) and a staining technique would therefore be a rapid means of screening for mutants in lipid production. However, staining was found to be too subjective, not quantitative and not specific enough to be suitable to screen for mutants in lipid production.

Bergman (1973) found that viability was 10 % of the untreated controls when Phycomyces heat-activated spores were treated with 0.1 mg/ml NTG in phosphate buffer at pH 7.5 for 30 min. This is comparable to the results in section 4.2.3, where 10 % survival was found after 30 min in C. curcubitarum. The spores were suspended in 0.1 mg/ml of NTG in distilled water as opposed to buffer.

Guerola et al. (1971) found that strains of E. coli selected for mutation in one gene by NTG had a high probability (of the order of 1 % per cistron) of being mutated in nearby genes. The occurrence of a high frequency of mutant strains of C. curcubitarum with both altered lipid and carotene production, after a single mutagenic treatment, could be a consequence of a similar process. A similar observation was made by Bergman (1973) who found a high frequency of mutant strains of Phycomyces having defects in both carotogenesis and phototropism. Bergman stated that double mutants of this

kind may introduce errors in biochemical and physiological studies by suggesting a common element for unrelated events. The fact that the increased carotogenesis and GLA production mutation in C. curcubitarum did not revert simultaneously, indicates that the mutation must have been a double mutation and confirms the observation made in physiological experiments that carotogenesis and lipid production are not linked (3.3.2 and 3.3.4). The best way of avoiding this would be to use a mutagen other than NTG.

CarC1 and carR1 were interesting mutants in that, had they been stable, they had potential of being used for the increased production of β-carotene by fermentation.

The method developed in section 2.3.3.1 using the HP method of extraction and analysis to screen for mutants was tried on the carC2 series of mutants and was found to be successful.

CHAPTER 5

CONCLUSION AND GENERAL DISCUSSION

There are two means available to improve the lipid production and GLA yield of C. curcubitarum, namely, 1) to optimise the environmental conditions and 2) to genetically manipulate the organism.

Not much more can be achieved by optimising the environmental conditions in liquid medium and on agar medium. Shaw's agar medium was shown to be optimum for the production of GLA by C. curcubitarum. All liquid medium experiments were carried out in shake flasks and improved yields were obtained when Chemap laboratory fermentors were used (A. van der Westhuisen, private communication). This may be due to increased aeration during the fermentation or even due to the anti-foam used. (Δ-6 desaturase activity was found to be stimulated by the presence of ionic detergents; Okayasu et al., 1979). Futher optimization could, therefore, be carried out in small-scale and large scale fermentations, but it is doubtful whether any considerable increase in the GLA yield will be achieved.

Maximum productivity requires that the organism used to be grown to or maintained at a high cell density (Ratledge, 1982). The maximum density is usually

- governed by the rate at which oxygen can be transferred into the fermentation broth (although other factors may restrict growth). A disadvantage of fungi is that they are generally difficult to grow and cell densities higher than 20g/l are rarely achieved. Yeasts, on the other hand, may be grown up to 80 to 100g/l and provide fermentation broths which are easier to mix.

Batch culture and fed-batch cultures are probably the least cost-effective means of cultivating organisms in that they fail to keep all the equipment fully used and are labour intensive processes (Ratledge, 1982). Success has, however, been achieved by researchers at National Chemical Products with small-scale and large scale fermentaion of C. curcubitarum. There is, therefore, no need to insist that a continuous fermentation process which requires sophisticated equipment be carried out.

The size of the innoculum does have a marked influence on the GLA yield by C. curcubitarum but this has not been formally researched and could be an area for further research.

The second alternative is to genetically manipulate the organism. Biotechnology of the oils and fats industry has been reviewed by Rattray (1984), who concludes that the most efficient applications of genetic engineering to oleaginous organisms will be, to a large extent, dependent on the availability of more detailed knowledge

of the biochemistry, physiology and genetics of the cell. In particular, more definite information on the genetic control of the biochemical processes associated with fat and oil synthesis is required. The complexities of the multiple gene/multiple gene product systems are generally recognised. Enormous improvements have, however, been made in industrial microbiology through the application of genetics and great improvements in yield due to mutation and selection have been achieved.

Only the basic groundwork has been covered in this thesis. A suitable assay medium, mutagen and screening technique have been found. This system was used succesfully to isolate mutants in GLA, lipid and carotene production. These mutants were unfortunately unstable. Ratledge (1984) reports that the reason for the spontaneous loss of oleaginicity in yeasts is obscure, but postulates that the logical reason for this phenomenon may lie in the enzymes which are easiliy lost from the genetic constitution of the organism. Nevertheless, mutants in GLA production were isolated and the chances of isolating a stable mutant do not seem remote.

A great deal of research into the biochemistry and genetics of lipid production in C. curcubitarum will, however, still have to be done.

In spite of the literature available on desaturases, it

It is apparent that much work has not been done on the desaturase system in fungi. Only the basic characteristics of the desaturases have been investigated. The precise mechanism of desaturase acivity is uncertain not only in fungi, but in mammals and plants as well. In addition, regulation of desaturase activity is important for microbial membrane functions because properties exhibited by polar lipids are primariy influenced by their constitutional fatty acids and even this area is not fully explored. Although yeast unsaturated fatty acid and desaturase mutants are known, these do not appear to have been used to investigate the mechanism and regulation of fatty acid desaturation. (Chopra & Khuller, 1984). Enzyme systems usually vary from species to species and research into the specific desaturase system in C. curcubitarum would have to be carried out.

The Δ-6 desaturase enzyme is difficult to isolate and purify and the enzyme assay is an extremely complicated procedure. The assay involves conversion of radioactively labled linoleic acid or linoleic acid-Coenzyme A to labled derivatives which must be extracted, esterified and separated by some form of chromatography before being counted (Safford et al., 1975; Okayasu et al. 1977;). Such an assay would not be suitable for screening of recombinant clones. Such clones would have to be detected by serological

techniques using antibodies or specific DNA probes. To achieve this the enzyme will have to be purified by Trition X-100 solubilization, DEAE-cellulose, CM-Sephadex and affinity chromatography on cytochrome b5-Sepharose (Okayasy et al., 1981). It should be possible for a biochemist to purify the enzyme and raise antibodies to it. Once the amino acid sequence has been determined, DNA probes can be synthesised and used for detecting the gene.

Enzyme inhibitors have been extensively used to study biochemical pathways. Potassium cyanide and p-chloromecuric benzene sulphonate and dithiothretol and β-mercapto ethanol are inhibitors of the desaturase. Their use in biochemical experiments may provide some answers to the function of GLA in C. curcubitarum.

The function of GLA in micro-organisms in general and in C. curcubitrum remains uncertain. Shaw (1966c) reviewed the function of polyunsaturated fatty acids in protists. It has been suggested that the function of alpha-linolenic acid is associated with chloroplasts and plant-like mode of life and that GLA and arachidonic acid are involved with that type of metabolism which depends on oxidative phosphorylation as its energy source (as in animals). Evolution towards animal forms is postulated to be accompanied by a loss of ability to synthesize α-linolenic acid and the corresponding disappearance of GLA from the plant line. Euglenids and

chrysomonads are capable of synthesizing both alpha-linolenic and gamma-linolenic acids and the metabolism of such protists has both plant- and animal-like characteristics. According to Shaw (1965) if the structure of gamma-linolenic acid has a fundamental function in photosynthetic plants, and if it is as important to the animal mode of life, then it may be that alpha-linolenic in non-photosynthtic higher fungi and GLA in non-motile Phycomyces, is vestigial. The fungi retain the genetic ability to biosynthesise these fatty acids even though the function of these fatty acids is relegated to one where the precise molecular structure is of little significance, such as energy storage. The GLA in the phospholipid fraction may play a role in electron transport as phospholipids are known to play an important role in mitochondrial electron transport.

Although a great deal of research effort would be required to achieve the ultimate aim of this project, the benefits of such research have tremendous potential. Firstly, if GLA remains as promising as it seems to be at present (and more data is accumulating to suggest that this is the case), the benefits would reach the general health of the world's population. In particular, GLA seems to be beneficial for aging diseases and by the year 2000 it is predicted that there will be 600 million aged people in the developed world. This could mean

epidemic proportions of chronic old age diseases which will dominate the health services.

Secondly, a greater understanding of the lipid biochemistry of C. curcubitarum may lead to increased knowledge of the role of fatty acids not only in micro-organisms but also in humans, where the importance of these compounds (in for instance arteriosclerosis and blood cholestrol levels) is only now being realised.

Finally, the world's resources of fats and oils will ultimately not be able to meet the needs of the escalating world population. Biotechnology in this industry may be the answer to the world's future oil demand.

APPENDIX

A: MEDIA PREPARATION

All medium was sterilised by autoclaving at 121°C for 20 min at 105 kPa.

Shaw's medium

Glucose	60g
Proteose Peptone (Lab M)	10g
KH_2PO_4	5g
Magnesium Sulphate	1g
Trace Element Solution	1ml
H_2O (distilled)	1L

The pH of the medium was adjusted to pH 5.55 prior to autoclaving.

Trace element solution:

Ferric sulphate	5g
Zink sulphate	1g
Copper sulphate	0.5g
Ammonium molybdate	0.1g
Manganese sulphate	0.1g
Boric acid	0.1g
Calcium hydroxide	0.1g
H_2O (distilled)	1L

The trace element solution was made up in two solutions, A and B, in order to prevent precipitation and stored at 4°C.

Solution A contained the calcium hydroxide, boric acid and ammonium molybdate.

Solution B contained the ferric sulphate, zink sulphate, manganese sulphate and copper sulphate.

MM1 (Minimal Medium 1)

Glucose	40g
Asparagine	2g
KH2PO4	0.5g
Magnesium sulphate	0.25g
Thiamine cloride	0.005g
(Agar	15g)
H2O (distilled)	1L

MM2 (Minimal Medium 2)

Glucose	20g
Mg SO4.7H2O	0.5g
KH2PO4	1g
Sodium succinate	5.0g
Ammonium sulphate	1.2g
H2O (distilled)	1L

The pH of the medium was adjusted to 6.0 - 6.5 with NaOH prior to autoclaving.

Assay medium

Yeast extract	5g
Glucose	20g
Agar (Oxiod No.1)	20g
H2O (distilled)	1L

The pH of the medium was adjusted to pH 4 after autoclaving, using Universal indicator strips (Merk) and 0.1 ml of 10 % tartaric acid was added to each 15 ml of the medium prior to pouring the plates.

GYE/Actidione agar medium

Glucose	10g
Yeast extract	3g
Agar	15g
H2O (distilled)	1L

An amount of 0.5 ml of a 2 % solution of actidione was added to 100ml of the medium prior to pouring the plates.

GYE/Novobiacin

Glucose	10g
Yeast extract	3g
Novobiacin	150mg
Agar	15g
H2O (distilled)	1L

B: - CALIBRATION MIXTURE FOR GLC ANALYSIS

Pentadecanoic acid	± 250mg
Myristic acid	± 100mg
Palmitic acid	± 300mg
Palmitoleic	± 100mg
Stearic acid	± 100mg
Oleic acid	± 200mg
Linoleic acid	± 200mg
γ-linolenic acid (GLA)	± 100mg
α-linolenic acid (ALA)	± 100mg

The calibration mixture was made up in 25 ml hexane and stored under N2 at 4 °C, in the dark. A 5 ml portion of this solution was esterified and used to calibrate the gas chromatograph.

C: MICROSCOPIC METHODS

The obsevations on the morphology of the colonies were made using a Zeiss-4 steriomicroscope.

Cell morphology was observed using a Zeiss standard microscope fitted with phase contrast optics.

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